



## ORIGINAL ARTICLE

# Liposome–DNA complexes infused intravenously inhibit tumor angiogenesis and elicit antitumor activity in dogs with soft tissue sarcoma

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Intravenous gene delivery using liposome–DNA complexes (LDC) has previously been shown to elicit antitumor activity, but only in rodent tumor models. Therefore, we conducted a study to determine in a large animal spontaneous tumor model whether intravenous infusions of LDC could target gene expression to cutaneous tumor tissues and whether repeated treatments had an effect on tumor growth or angiogenesis. A total of 13 dogs with cutaneous soft tissue sarcomas were enrolled in the study and were randomized to receive a series of 6 weekly infusions of LDC containing either canine endostatin DNA or DNA encoding an irrelevant gene (luciferase). Serial tumor biopsies were obtained to assess transgene expression, tumor microvessel density (MVD), and intratumoral leukocyte inflammatory responses. We found that intravenous infusion of LDC did not result in detectable gene expression in cutaneous tumor tissues. However, two of 13 treated dogs had objective tumor responses and eight dogs had stable disease during the treatment period. In addition, a significant decrease in tumor MVD was noted in six of 12 treated dogs at the completion of six treatments. These results suggest that intravenous infusions of LDC may elicit nonspecific antitumor activity and inhibit tumor angiogenesis.

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## Introduction

Tumor angiogenesis inhibition continues to hold great promise as an approach to controlling tumor growth.<sup>1–7</sup> Moreover, several recent clinical trials of angiogenesis inhibitors such as VEGF antagonists have shown positive results in patients.<sup>8–12</sup> A variety of different inhibitors of angiogenesis, including synthetic molecules and endogenous antiangiogenic substances, have been described, including endostatin.<sup>3,13,14</sup> However, early clinical trials of recombinant endostatin failed to demonstrate convincing antitumor activity, although the compound was well tolerated.<sup>15,16</sup> The lack of endostatin activity in the early

trials was attributed in part to the inability to produce sustained systemic levels in the bloodstream.<sup>3,5,14</sup>

Systemic gene delivery offers an alternative to repeated administration of protein drugs. For example, use of systemic gene delivery to express endostatin or other angiogenesis inhibitors to control tumor growth has been reported previously in mouse models.<sup>17–33</sup> However, potential hurdles to applying systemic gene therapy in humans include achieving efficient *in vivo* transfection, attaining durable *in vivo* gene expression, and administering the gene delivery vectors repeatedly.<sup>2,17,23</sup> The use of nonviral gene delivery systems, based principally on liposome–plasmid DNA-based systems, may help address some of these problems, particularly the issue of repeated gene delivery. Use of cationic liposome–DNA complexes (LDC) for inhibition of tumor angiogenesis has the added potential advantage that the complexes have been shown to preferentially target the angiogenic endothelium in tumor tissues *in vivo*, an effect that may be enhanced by preinjection of protamine.<sup>34,35</sup> Thus, it may be possible to express antiangiogenic genes directly in angiogenic blood

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vessels of tumors by intravenous administration of LDC. The LDC themselves have also been shown to elicit strong activation of innate immunity and release of several potent antiangiogenic cytokines, including IL-12, IFN- $\gamma$ , and IFN- $\alpha$ .<sup>36-38</sup> Thus, use of LDC for delivery of angiogenesis-inhibiting genes may inhibit tumor angiogenesis by eliciting both gene-specific and gene nonspecific effects.

A key question in the gene therapy field is whether intravenous infusion of LDC could target gene delivery and expression directly to tumor tissues in extrapulmonary sites such as tumors in cutaneous tissues. If successful, such an approach would have considerable appeal for targeting of metastatic tumors not accessible to direct injection. Therefore, we designed a study in a large animal tumor model to determine whether intravenous infusions of LDC could generate transgene expression in cutaneous tumor tissues and also inhibit tumor angiogenesis. Pet dogs with large, spontaneous soft tissue sarcomas were utilized in this study, as this tumor in dogs is refractory to most conventional therapies other than surgical excision and is also amenable to repeated tumor biopsies.<sup>39-41</sup> The study was designed in part to compare tumor responses in dogs infused with canine endostatin DNA to dogs treated with control (luciferase) DNA to determine whether endostatin gene delivery was effective in inhibiting tumor angiogenesis. The control luciferase vector was included to account for the potential nonspecific antiangiogenic and antitumor effects of the LDC vector itself.<sup>36,37</sup> The dose of LDC delivered to dogs in this study was based on results of a prior Phase I trial of intravenous gene delivery in dogs with lung tumor metastases.<sup>42</sup> The study end points were gene expression in tumor tissues, the effects of LDC infusion on tumor angiogenesis, and the effects of LDC infusion on intratumoral inflammatory responses. The effects of treatment on clinical parameters and circulating levels of VEGF and basic FGF were also assessed.

We found that intravenous infusion of LDC did not result in detectable levels of transgene expression in any tumor tissues, even using a very sensitive detection assay for gene expression. However, we did find that repeated intravenous infusions of LDC resulted in significant inhibition of tumor angiogenesis in more than half of treated dogs and also elicited a strong infiltrate of CD8 + T cells into some tumors. These effects were independent of the gene delivered, indicative of a nonspecific vector effect. Our results therefore suggest that intravenous delivery using the current LDC formulations may not be effective for systemic gene delivery to tumors, but the LDC infusion may, however, inhibit tumor angiogenesis and elicit antitumor immunity in a gene nonspecific fashion.

## Materials and methods

*Plasmid constructs and assessment of in vitro expression*  
The pMB517 canine endostatin expression vector was prepared by cloning the canine endostatin cDNA into a previously described pMB75.6 plasmid expression vector.

The pMB75.6 expression vector utilized the CMV immediate-early promoter-enhancer region, a synthetic intron (pGL3) immediately upstream of the start site, an SV40 early poly(A) site, and the kanamycin resistance gene, as described previously.<sup>43</sup> The cDNA for canine endostatin was synthesized (Operon Inc., Huntsville, AL) based on published sequence data and sequence information provided by Entremed Inc. (Rockville, MD). The canine endostatin cDNA was also linked to the human Ig secretory signal to facilitate secretion of the endostatin gene product. The luciferase cDNA was kindly provided by R Debs (California Pacific Research Institute) and was also cloned into the pMB75.6 expression vector to produce the pMB408 vector. Expression of the canine endostatin gene construct was assessed by transfecting a canine melanoma cell line with the plasmid pMB517 construct or with an irrelevant plasmid (pMB408), using lipofection. Immunohistochemistry was performed using a polyclonal rabbit antiserum raised against canine endostatin (Cytimmune Sciences, Rockville, MD) to demonstrate endostatin expression in the canine cell line. Briefly, fixed cells were incubated with appropriately diluted primary antibody for 20 min at room temperature, washed, incubated with biotinylated anti-rabbit antisera (Jackson ImmunoResearch, West Grove, PA), then washed and incubated with HRP-conjugated streptavidin (Jackson ImmunoResearch), then washed and incubated with diaminobenzidine substrate (Sigma-Aldrich, St Louis, MO), then briefly counterstained with hematoxylin and coverslipped. Controls included use of nonimmune rabbit Ig and immunostaining of nontransfected cells and cells transfected with irrelevant plasmid DNA. Immunostained cells were photographed using an Olympus microscope.

Secretion of endostatin was assessed by commercial ELISA assay (Cytimmune). Briefly, cells in six-well plates were transfected by lipofection, then rinsed and cultured for an additional 24 h. Supernatants were collected and assayed for endostatin production using a commercial canine endostatin-specific ELISA (Cytimmune), according to the manufacturer's directions. Supernatants from mock-transfected cells were used as controls. Endostatin concentrations were determined by comparison to a standard curve generated with recombinant canine endostatin (Cytimmune). The biological activity of the secreted canine endostatin construct was also assessed using an endothelial cell migration inhibition assay. Briefly, nontransformed murine capillary endothelial cells (CD3 cells, kindly provided by Clement Diglio, Wayne State University) were grown to confluence in 150 mm tissue culture dishes (Corning). Then, using a sharp blade, a scratch was made through the center of the culture to produce a sharply demarcated zone denuded of viable cells. The plates were washed to remove detached cells, then supernatants from transiently transfected canine melanoma cells were added at a 1:1 dilution to the CD3 cells, which were then cultured for an additional 24 h. Then, the cells were fixed in methanol and counterstained with hematoxylin. Using a dissecting microscope, the number of endothelial cells that had migrated into the

denuded zone of the plate were counted in 10–20 high-power fields per plate to determine the mean number of migrating cells. Controls included cells cultured with nontransfected CHO supernatants and with supernatants from CHO cells transfected with irrelevant plasmid DNA.

#### *DNA and liposome preparation*

Plasmid DNA was propagated in *Escherichia coli* and isolated by alkaline lysis, followed by column chromatography, as reported previously.<sup>44</sup> The endotoxin content of the DNA was determined by LAL assay (Biowhittaker, Walkersville, MD) and was found to be less than 0.25 EU/ $\mu$ g DNA. The stock DNA solution (prepared in a 10 mM solution of Tris-HCl (2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride), pH 8.0 at 5.0 mg/ml) was diluted to 0.625 mg/ml with 5% w/v dextrose (D5W). Liposomes were prepared by dissolving cholesterol (Sigma Chemical Co., St Louis, MO) and the cationic lipid DOTIM (octadecenylolxy{ethyl-2-heptadecenyl-3 hydroxyethyl}imidazolium chloride) in chloroform (EM Sciences, Gibbstown, NJ) at equimolar concentrations in a 3-l round-bottom flask. The clear solution was rotated on a Buchi rotovapor R-134 at 30°C for 30 min and hydrated in 5% w/v dextrose (McGraw, Irvine, CA) to produce a nominal concentration of 40 mM. The liposome dispersion was extruded through successive filters (0.4, 0.2, 0.1, and 0.05  $\mu$ m) to obtain a vesicle size of approximately 100 nm as measured by a Nicomp C-370 particle sizer. Immediately prior to infusion, CLDC were prepared by first dissolving liposomes in 5% dextrose in water at a concentration of 100  $\mu$ l liposomes per 1 ml of 5% dextrose solution, as described previously.<sup>45</sup> Next, the plasmid DNA was added to the liposome solution at a final concentration of 100  $\mu$ g DNA/ml solution and complexes were formed by gentle pipetting. The LDC were infused intravenously within 15 min of preparation.

#### *Preparation of BODIPY-labeled LDC*

Fluorescently labeled cationic liposomes were prepared by addition of BODIPY-labeled cholesteryl (Molecular Probes, Eugene, OR) to the unlabeled cholesterol and DOTIM at the time the liposomes were prepared. The BODIPY-labeled cholesterol was added to unlabeled cholesterol at a 1:5 M ratio and the labeled and unlabeled cholesterol was added at a 1:1 M ratio with DOTIM, then dried down. The labeled liposomes were rehydrated as described above, then used to prepare LDC, which were infused intravenously as for unlabeled LDC.

#### *Trial design*

Pet dogs with biopsy-confirmed cutaneous soft tissue sarcomas were eligible for entry into the study. These studies were approved by the Institutional Animal Care and Use Committees at the National Jewish Medical and Research Center and at Colorado State University. Dogs with concurrent medical diseases or those receiving concurrent immunosuppressive therapy or prior radiation therapy to the site of the tumor were excluded from entry. Dogs were randomized by block randomization scheme (Research Randomizer) to receive treatment with either

canine endostatin DNA or with irrelevant (luciferase) DNA. Investigators administering the treatment were blinded as to treatment groups. Dogs were treated once weekly with an intravenous infusion of LDC prepared with either endostatin or luciferase plasmid DNA. A total of six treatments were administered weekly for 6 weeks. The tumor was then surgically removed 3 days after the last treatment and analyzed by routine histology and immunohistochemistry.

#### *Treatment and monitoring*

Prior to treatment, all dogs had an indwelling intravenous catheter placed in either the cephalic vein or the medial saphenous vein, without sedation. Using a syringe pump (Harvard Apparatus, Holliston, MA), the LDC solution was infused as a continuous rate infusion over a 90-min period. Each dose of LDC consisted of 20  $\mu$ g/kg body weight of plasmid DNA. This dose of LDC and rate of infusion was selected based on preclinical studies of intravenous LDC infusion and gene expression in rabbits (D Liggitt, unpublished data) and on the results of a phase I study completed previously in pet dogs with lung tumor metastases.<sup>42</sup> Thus, for a typical 25 kg dog receiving a 20  $\mu$ g/kg dose of plasmid DNA, the total DNA dose delivered was 500  $\mu$ g and the infusion rate was 55  $\mu$ l/min. For the first 24 h after the first infusion, dogs were kept under observation in the hospital and body temperature and respirations were monitored every 2 h. For subsequent treatments, dogs were monitored for the first 4–6 h postinfusion, then returned home with their owners.

Routine complete blood counts and serum biochemistry analysis were performed before treatment, 24 h after the first treatment, and again on weeks 1, 4, and 6 of the study. Tumor measurements in three dimensions were obtained using calipers at each treatment and the tumor volume was calculated. Tumor responses were classified as partial response if tumor volume decreased by >50% from pretreatment measurements and complete response if the tumor regressed completely and was no longer detectable. Stable disease was defined as tumor volume that did not increase or decrease by >50% from starting values, whereas progressive disease was defined as tumor volume that increased >50% from starting values.

#### *Tumor biopsy and assessment of tumor microvessel density and inflammation scores*

Tumor biopsies were obtained under local anesthesia by wedge biopsy immediately prior to the first treatment, at 24 h after the first treatment, at 7 days after the first treatment, and on week 6 of the study. Portions of the biopsy were flash-frozen for RNA analysis (see below) while the remainder of tumor tissue was embedded in Tissue-Tek OCT compound (TedPella Inc., Redding, CA) and snap-frozen in isopentane. Tissues were cryosectioned to a thickness of 4  $\mu$ m and adhered to glass slides. For identification of tumor microvessels and angiogenic endothelium, a mAb specific for human CD146 (clone PIH12, Chemicon, Temecula, CA) that cross reacts with dog endothelium was utilized.<sup>46</sup> After fixation in acetone

and blocking nonspecific binding, tissues were incubated first with the primary antibody, followed by donkey anti-mouse IgG antiserum (Jackson ImmunoResearch), then with streptavidin-HRP and AEC substrate (Vector Laboratories, Burlingame, CA), followed by hematoxylin counterstain. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies. Staining of normal canine spleen tissues was utilized for positive control for microvessel density (MVD) and inflammation assays.

For quantitation of tumor MVD, four photomicrographs at  $\times 10$  magnification were obtained by digital camera (Leica Microscope equipped with digital camera with SPOT Advanced Imaging software). Utilizing Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC), the photomicrographs were converted to binary images and the number of microvessels per section was determined digitally. MVD values for each tumor biopsy were calculated based on the average number of vessels for the four  $\times 10$  fields.

Pre- and post-treatment tumor biopsies were also immunostained for detection of tumor-infiltrating leukocytes (TIL). Monoclonal antibodies specific for canine CD4 (clone YKIX 302.9, Serotec, Oxford, UK), canine CD8 (clone YCATE 55.9, Serotec), and canine CD11b (clone CA16.3E10, Serotec) were utilized for these studies. Appropriately diluted antibodies were incubated with acetone fixed sections as described above, along with appropriate negative controls. The degree of leukocyte infiltration was assessed by examining at least 10 random high-power fields ( $\times 40$ ) fields per slide. Semi-quantitative scores of the degree of leukocyte infiltration were assigned, based on the following system (0 = no leukocytes found; 1 = 1–5 cells/high-power field (hpf); 2 = 6–10 cells/hpf, 3 = 11–15 cells/hpf; and 4  $\geq$  15 cells/hpf).

#### *Quantitative RT-PCR assay for transgene and cytokine gene expression in tumor tissues*

Quantitative RT-PCR was utilized to detect transgene expression in tumor tissues, as previously described.<sup>43</sup> Tumor biopsies were obtained pretreatment and again 24 h after the first LDC infusion and were then snap-frozen in isopentane in dry ice and stored at  $-80^{\circ}\text{C}$  prior to analysis. After thawing, tumor biopsies were immersed in RNA storage solution (RNA Later, Invitrogen, Carlsbad, CA) and then pulverized using disposable tissue homogenizers. The RNA was then extracted, reverse transcribed to cDNA, treated with DNase, and amplified using a set of primers specific for a synthetic intron (pgI) incorporated between the promoter and the start site of the gene expression vector pMB75.6 used in these experiments.<sup>43</sup> The primer and probe combinations were those described previously and spanned the 5'UTR region of the expression vector. This strategy allowed amplification of the synthetic intron sequence from vector-encoded mRNA and avoided contamination with endogenous transcripts. This assay was found to be sensitive to one copy of template DNA when template DNA was added to control tissue homogenates (data not

shown). Quantitation of mRNA transcripts was performed on an ABI Prism 7000 cycle sequencer and the level of mRNA expression was indexed to mRNA levels of a housekeeping gene (GAPDH).

#### *Measurement of plasma VEGF and basic FGF concentrations*

Plasma was collected from each dog prior to treatment, and again at 8, 12, and 24 h after the first treatment, and then again at weeks 1, 2, and 6 of treatment. Plasma was stored frozen at  $-80^{\circ}\text{C}$  prior to analysis. Plasma VEGF and basic FGF concentrations were determined utilizing commercial ELISA assays (R&D Systems, Minneapolis, MN), according to the manufacturer's directions. Both of these assays have been previously validated for use in detecting canine VEGF and canine basic FGF.<sup>47–49</sup>

#### *Endothelial cell toxicity assay*

Nontransformed mouse capillary endothelial cells (a gift of C Diglio, Wayne State University) were used to assess endothelial toxicity of LDC. Endothelial cells were plated at a density of  $5 \times 10^4$  cells/well in 96-well plates in complete medium (minimum essential medium (Invitrogen Corp., Grand Island, NY), supplemented with 10% fetal bovine serum (Sigma, St Louis, MO), penicillin-streptomycin, L-glutamine, essential amino acids, and nonessential amino acids (Gibco, Grand Island, NY)). Cells in quadruplicate wells were incubated with serial dilutions of LDC prepared with noncoding plasmid DNA, at the same concentration as used for *in vivo* infusions. After addition of LDC, the cells were incubated for 24 h and then cell viability was determined by MTT assay. Briefly, cells were incubated with MTT solution (Sigma) for 2 h, lysed with acidified isopropanol, then the optical density determined by optical scanner (Multiscan Ascent; Thermo Labsystems, Franklin, MA). Cell survival was calculated by determining the mean OD 570 nm for quadruplicate wells.

#### *Tumor inhibition assay*

The ability of LDC to elicit cytokines capable of inhibiting fibrosarcoma tumor cell growth *in vitro* was assessed using spleen supernatants from mice injected intravenously with LDC prepared with noncoding DNA. Briefly, supernatants were prepared by overnight culture of spleen cells obtained from LDC-injected mice (four mice per group) or untreated control mice 3 h after intravenous injection of LDC, at a dose of  $10 \mu\text{g}$  DNA per mouse. Supernatants were collected and stored frozen at  $-80^{\circ}\text{C}$  prior to assay. Murine fibrosarcoma cells (MCA 2.1; derived in our laboratory by methylcholanthrene-induced carcinogenesis in the skin of mice) were cultured in 96-well plates at a density of  $5 \times 10^3$ /well and incubated with serial dilutions of supernatants from spleen cells of control or LDC-injected mice to assess their effects on tumor cell viability and proliferation. After 24 h of incubation of quadruplicate wells with serial dilutions of spleen cell supernatants, the number of viable cells in

each well was determined by MTT assay as described above.

### Statistical analysis

Statistical differences in tumor microvessel densities were assessed using a univariate nonpaired *t*-test to compare pretreatment tumor MVD to tumor MVD at other time points. Differences in inflammatory pre- and post-treatment inflammatory infiltrates in tumor tissues were assessed by paired *t*-test. Comparison of differences in tumor cell viability and endothelial cell viability following treatment with spleen cell supernatants or LDC, respectively, was made by paired *t*-test.

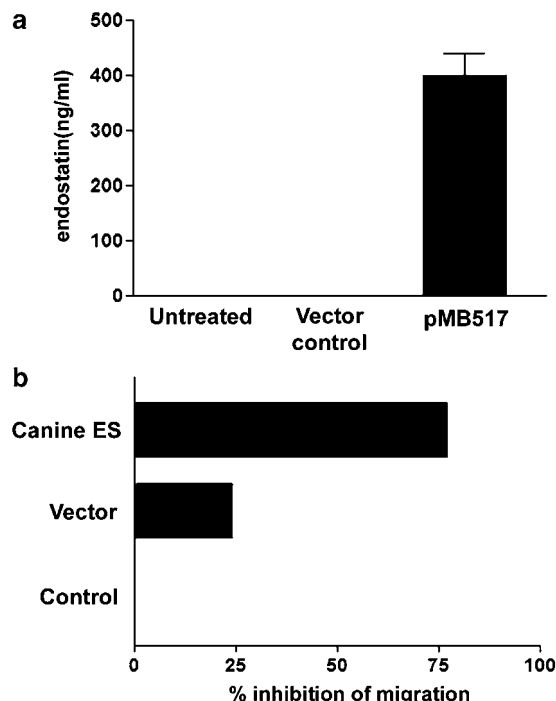
## Results

### *In vitro* expression of canine endostatin cDNA

To assess expression of canine endostatin by the pMB5176 vector, cell lines were transfected with plasmid DNA and expression was quantitated by canine endostatin ELISA and by immunohistochemistry. Production of secreted endostatin was detected in supernatants of cells transfected with the canine endostatin construct, but not in supernatants from cells transfected with irrelevant DNA (Figure 1a). Canine endostatin activity was also detected by immunohistochemistry in transfected canine melanoma cells (data not shown). Production of biologically active endostatin by transfected cells was demonstrated by inhibition of endothelial cell migration (Figure 1b). Therefore, the pMB517 construct produced and secreted biologically active canine endostatin following transfection of canine cell lines.

### Treatment responses and tumor responses following treatment with LDC encoding endostatin or luciferase DNA

Dogs enrolled in this study all had soft tissue sarcomas located in the skin, where they were accessible for measurement and biopsy. A total of 13 dogs were enrolled in the study and received at least one infusion of LDC (Table 1). One dog (number 13; Table 1) was withdrawn shortly after entering the study due to progressive tumor growth, while the rest of the dogs received the full course of six treatments. Seven dogs were treated with LDC containing the endostatin gene and six received infusions of LDC containing the luciferase gene. All dogs tolerated the LDC infusions well. Most dogs developed fever within 6 h of beginning the infusion, and most dogs also developed transient lymphopenia for the first 24 h following treatment, consistent with what has been reported previously (data not shown).<sup>42</sup> Significant changes in other hematologic or biochemical parameters were not observed during the course of the study in any treated dogs (data not shown). For the 13 dogs treated in the study, objective tumor responses were observed in two animals: One dog experienced complete tumor regression, one dog had partial tumor regression, while eight dogs had stable disease and three dogs experienced progressive tumor growth during the course of the 6-week study.



**Figure 1** Secretion of canine endostatin by transfected cells and demonstration of biological activity. (a) To assess the ability of the pMB517 vector to produce and secrete canine endostatin, canine melanoma cells in triplicate wells were transfected *in vitro* using lipofection. Controls included nontransfected cells and cells transfected with a luciferase plasmid. At 24 h after transfection, supernatants were collected and assayed for the presence of canine endostatin protein by specific ELISA (Cytimmune). Cells transfected with pMB517 vector produced substantial amounts of endostatin. (b) The biological activity of the secreted endostatin was assessed using an endothelial migration assay as described in Materials and Methods. Supernatants from canine melanoma cells transfected with the canine endostatin construct (pMB517) or a luciferase control plasmid by lipofection were collected 24 h post-transfection and diluted 1:1 with medium and the effects on endothelial migration were assessed. Incubation with supernatants from pMB517-transfected cells resulted in significant ( $P < 0.05$ ) inhibition of endothelial cell migration, compared to control supernatants. Similar results were obtained in one additional experiment.

### Tumor MVD changes following LDC infusion

Tumor biopsies were processed by immunohistochemistry for assessment of tumor MVD, using the PIH12 antibody. An example of tumor MVD changes in pre- and post-treatment tumor biopsies is illustrated in Figure 2, as is the conversion of the initial digital images into binary images for quantitation of MVD. The values for tumor MVD were determined from pre-treatment biopsies and from biopsies taken 7 days after the first infusion of LDC and again at day 42 of the study (Figure 3 and Table 1). It should be noted that although there was a wide range of starting MVD values between tumors in different dogs, within a given dog the tumor MVD values were relatively consistent from biopsy to biopsy, suggesting that sample biopsy site variability was not a major factor in the MVD analysis. When MVD values for pretreatment biopsies

**Table 1** Tumor responses and microvessel vessel density responses to intravenous infusion of LDC in dogs with soft tissue sarcomas

Dog	Treatment	Tumor response	MVD
1	ES	SD	Decreased
2	ES	SD	Decreased
3	ES	SD	Unchanged
4	ES	SD	Decreased
5	ES	PD	Unchanged
6	ES	SD	Unchanged
7	ES	SD	Increased
8	Lucif	CR	Decreased
9	Lucif	PD	Decreased
10	Lucif	SD	Decreased
11	Lucif	SD	Unchanged
12	Lucif	PR	Unchanged
13	Lucif	PD	NA

Clinical data for 13 dogs with soft tissue sarcoma enrolled in a clinical study of intravenous LDC gene delivery. Of the 13 dogs enrolled, seven dogs were treated with LDC containing canine endostatin plasmid DNA (ES) and six dogs were treated with LDC containing luciferase DNA (lucif). Tumor responses were classified as CR (complete regression of all visible tumor), PR (partial regression; >50% decrease in overall tumor volume), SD (stable disease, no increase or decrease in tumor volume >50%), and PD (progressive disease, >50% increase in tumor volume). Tumor microvessel density (MVD) was considered decreased if the MVD was decreased significantly ( $P < 0.05$ ) at the completion of treatment compared to pretreatment MVD, or increased if the MVD increased significantly at the completion of treatment compared to pretreatment MVD. The tumor MVD was considered unchanged if it did not change significantly between the beginning and the end of treatment. NA = not available for analysis.

were compared to those of post-treatment biopsies, we found that tumor MVD was decreased significantly by day 42 in six of the 12 treated dogs where post-treatment biopsies were available. Two dogs initially experienced a significant decrease in tumor MVD at day 7 of the study, but by day 42 the tumor MVD had increased again to values that were not statistically different from pretreatment values. Overall, five dogs had no change in tumor MVD between the beginning and end of study, while one dog developed a significant increase in tumor MVD at the end of the study. When tumor MVD responses were compared between dogs, there was no statistically significant difference between response rates in dogs treated with endostatin DNA and dogs treated with luciferase DNA.

#### Assessment of transgene expression in tumor tissues following *in vivo* gene delivery

Transgene expression was assessed in pretreatment and 24-h post-treatment tumor biopsies from study dogs. For this analysis, a sensitive RT-PCR assay was utilized, based on the unique design of the expression plasmid. *In vitro* plasmid add-back studies revealed that the assay used was sensitive down to the level of approximately 10 gene copies (data not shown). When 24-h post-treatment

tumor biopsies were analyzed from the treated dogs, transgene expression was not detected in any of the samples analyzed. The failure to detect transgene expression was not, however, due to RNA degradation, as GAPDH mRNA was detectable in all the samples. Serum from several dogs obtained at 8 or 24 h after their first LDC infusion was also evaluated by endostatin ELISA for the presence of circulating canine endostatin activity, but in no case was endostatin detected (data not shown). Based on these results, it appeared therefore that intravenous infusion of LDC was not an effective means of generating transgene expression in tumor tissues of dogs with established tumors. It is still possible, however, that low levels of transgene expression may have occurred in normal tissues such as the lung. However, given that tumor inhibition by endostatin generally requires exposure to high doses *in vivo*, the amount theoretically produced in the lungs and released into circulation is unlikely to have been biologically relevant.<sup>14,15</sup>

#### Localization of labeled LDC in tumor tissues

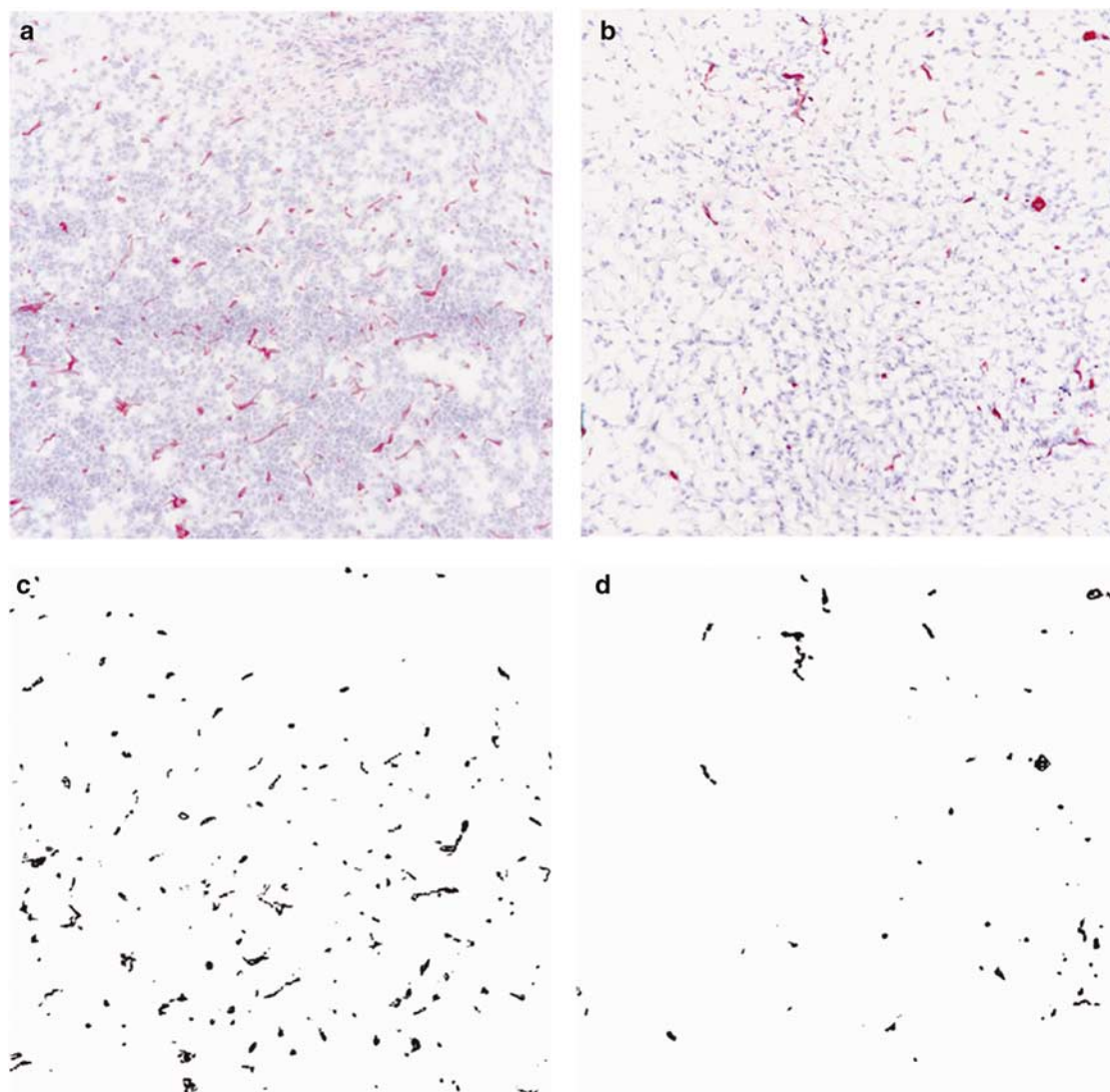
To determine whether the LDC were in fact delivered to the tumors, LDC labeled with the fluorescent dye BODIPY were infused in one dog and tumor biopsies were obtained pretreatment and again 120 min postinfusion. When the tumor tissues were examined by fluorescence microscopy, we observed numerous cells containing BODIPY + LDC within the tumor tissues (Figure 4). The location of the labeled LDC suggested that they were present in or adjacent to blood vessels, although this could not be confirmed by dual labeling studies. Thus, intravenous infusion of LDC resulted in delivery of the complexes to tumor tissues.

#### Infiltration of leukocytes in tumor tissues following LDC infusion

To assess the response of TIL to LDC infusion, pretreatment and day 42 tumor biopsies were immunostained for quantitation of numbers of CD4+ and CD8+ T cells and CD11b+ macrophages in tumor tissues (Figure 5a). The numbers of CD4+ T cells and CD11b+ macrophages were not significantly different in tumor tissues following LDC treatment when compared to pretreatment values. However, four dogs developed an increase in CD8+ T cells in tumor tissues following treatment, whereas CD8+ T cells were not observed in any tumor biopsies obtained prior to the start of treatment. In one treated dog, there was a dramatic increase in infiltrating CD8+ T cells following treatment (Figure 5b and c). Thus, infusion of LDC appeared to trigger an influx of CD8+ T cells into tumor tissues in some dogs, although the functional status of T cells was not assessed in this study.

#### LDC elicit endothelial cell cytotoxicity

The preceding results suggested that most of the effects observed in the study (tumor regression or tumor stasis, decrease in MVD, infiltration of CD8 T cells) are likely to have been elicited by the vector itself. These non-specific vector-induced effects could have included direct



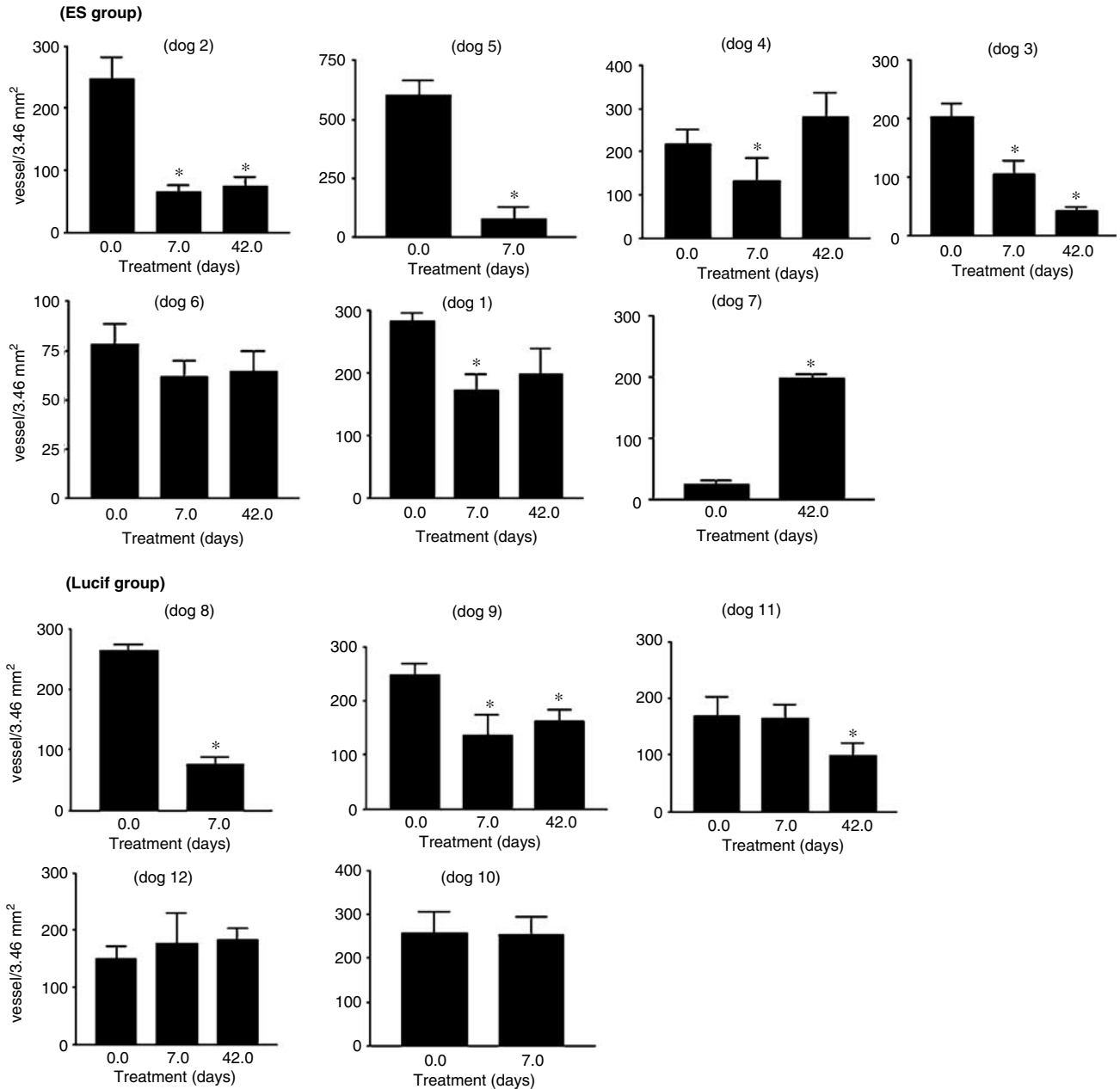
**Figure 2** Assessment of tumor MVD by immunohistochemistry and digital analysis. Tumor biopsies were obtained from a dog prior to treatment and again after six infusions of LDC encoding the canine endostatin gene. Cryosectioned tissues were then immunostained with the P1H12 antibody for quantitation of MVD, as described in Materials and methods. Multiple low-power fields from each biopsy were digitally photographed and the images converted to binary images (Reindeer Graphics) for quantitation of MVD. Pre-treatment tumor biopsies demonstrating tumor microvessels by light microscopy (vessels are red, panel a) and again after the image was converted to a binary image for quantitation (vessels are black; panel c) as shown. In panel b (and binary image in panel d), post-treatment biopsies demonstrated a significant reduction in tumor MVD.

endothelial cell toxicity by LDC or inhibition of tumor growth by cytokines elicited by the immune stimulatory effects of LDC.<sup>36,50</sup> To assess direct effects of LDC on endothelial cells, primary cultures of mouse endothelial cells were used, as normal canine endothelial cell lines were not available. LDC are known to be toxic to other cells *in vitro*, but their effects on endothelial cells have not been reported previously. We found that endothelial cells were in fact quite sensitive to killing by LDC. For example, incubation of endothelial cells with doses of LDC as low as 0.25  $\mu\text{g}/\text{ml}$  elicited substantial killing (Figure 6a). Thus, it is possible that inhibition of tumor angiogenesis by LDC infusions may have been mediated

in part by direct endothelial cell killing, particularly in dogs where significant inhibition of angiogenesis was observed as early as 7 days following the first LDC infusion (Figure 3). In support of this theory, LDC could be localized in tumor tissues within 2 h of infusion (Figure 4).

#### *Infusion of LDC triggers release of cytokines with tumor inhibitory activity*

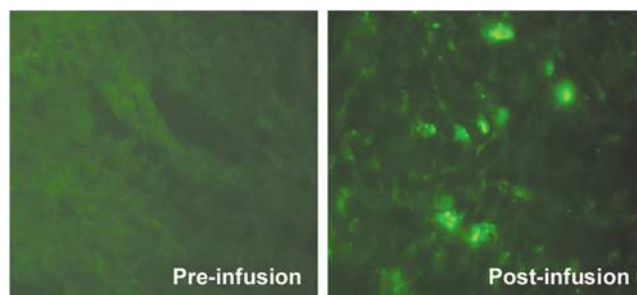
Two dogs in this study experienced significant tumor regression and eight dogs experienced lack of tumor growth over the 6-week treatment period. Although angiogenesis inhibition might account for some of this



**Figure 3** Tumor angiogenic responses to intravenous infusions of endostatin or luciferase LDC. Serial tumor biopsies were obtained from 12 dogs that each received a series of six infusions of LDC containing either canine endostatin plasmid DNA (ES group) or luciferase plasmid DNA (Lucif group). Tumor biopsies were obtained before treatment, at day 7 after the first treatment, and on day 42 after six treatments had been administered. The tumor biopsies were cryosectioned and immunostained for identification of tumor microvessels by immunohistochemistry and tumor MVD was quantitated for each biopsy as described in Materials and methods, and the mean ( $\pm$ s.e.) MVD for each biopsy was plotted. (ES group) Assessment of tumor MVD in seven dogs treated with six infusions of LDC encoding the canine endostatin cDNA. (Lucif group) Assessment of tumor MVD in five dogs treated with six infusions of LDC encoding luciferase DNA (\* $P < 0.05$ , for mean VD value, compared to pretreatment MVD).

effect, we also observed that tumor responses were not always correlated with changes in MVD. For example, one dog with a partial tumor response had no change in MVD, while MVD was unchanged in three dogs with stable disease. Thus, it was also possible that immunological mechanisms might also account in part for the antitumor activity observed. For example, previous

studies in mice have shown that LDC injected intravenously trigger potent release of cytokines with antitumor activity, including IL-12, IFN- $\gamma$ , IFN- $\alpha$ , and TNF.<sup>36,37,50</sup> Studies in mice have also demonstrated that a large portion of the infused complexes localize to the spleen, where they are taken up by macrophages and induce immune activation.<sup>34</sup> Thus, cytokine production by the



**Figure 4** Localization of fluorescently labeled LDC in tumor tissues. To determine whether LDC were taken up by tumor tissues following intravenous infusion, LDC were prepared using BODIPY-labeled liposomes and infused intravenously into a dog with soft tissue sarcoma, as described in Materials and methods. A pretreatment tumor biopsy was obtained, the labeled LDC were then infused over 90 and 60 min later, a second tumor biopsy was obtained. Tissues were frozen, then cryosectioned and examined by fluorescence microscopy. Several cells with BODIPY+ material are visualized in the postinfusion biopsy sample.

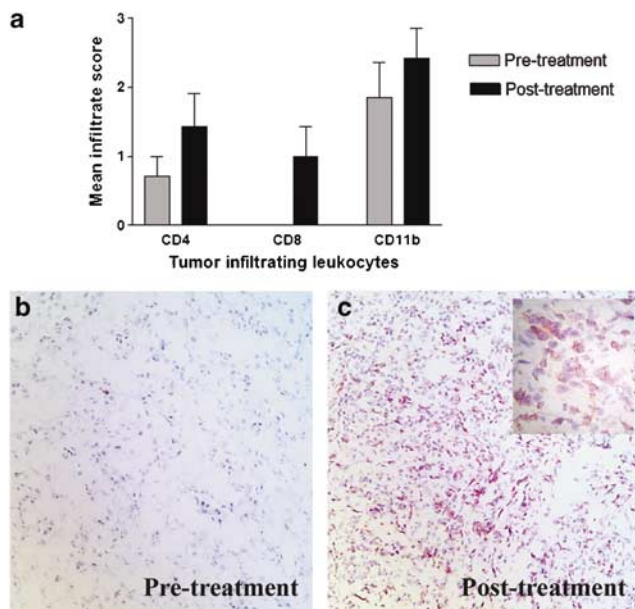
spleen in response to LDC infusion could trigger immune responses capable of suppressing tumor growth at distant sites. To address this question, we determined whether cytokines released from spleen tissues in mice following intravenous injection of LDC could also inhibit tumor growth *in vitro*. For these studies, a murine fibrosarcoma cell line (similar to the tumor type studied in dogs in this study) was used to assess antitumor responses. Addition of supernatants from spleens of LDC-injected mice, but not supernatants from control spleens, inhibited fibrosarcoma cell growth *in vitro* (Figure 6b). These results therefore suggest that LDC-triggered systemic release of cytokines with antitumor activity would be capable of inhibiting tumor cell growth *in vivo*. In fact, such antitumor activity of LDC has been demonstrated previously in several different tumor models in mice.<sup>36,50</sup>

#### Response of plasma VEGF and basic FGF concentrations to LDC infusion

To determine whether infusions of LDC might have inhibited tumor angiogenesis by decreasing circulating concentrations of key proangiogenic cytokines, VEGF and basic FGF concentrations in plasma were quantitated before, during, and after LDC infusions, using cross-reactive ELISA assays (see Materials and methods). Significant changes in the concentrations of VEGF or basic FGF were not observed in plasma samples collected during treatment, as compared to pretreatment plasma VEGF or FGF concentrations (data not shown). Thus, any effects of LDC infusion on tumor angiogenesis were most likely not due to suppression of circulating concentration of key proangiogenic cytokines.

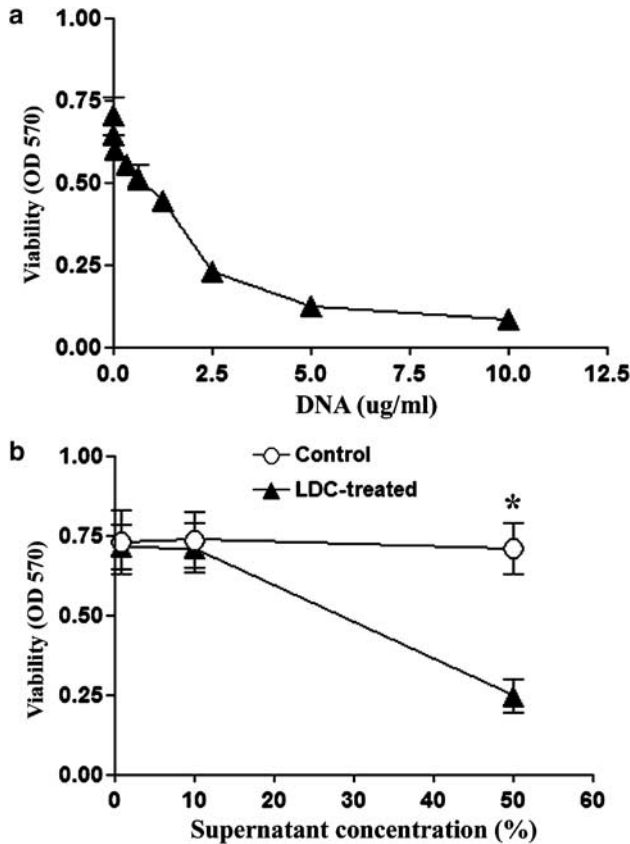
#### Discussion

This study was designed primarily to address two questions. Firstly, could intravenous infusions of LDC



**Figure 5** Leukocyte inflammatory responses in tumors before and after LDC infusion. Tumor biopsies were obtained from 12 dogs with soft tissue sarcoma prior to treatment and after six treatments with LDC. Tumors were cryosectioned and sections were immunostained with mAbs specific for canine CD4, CD8, or CD11b, as described in Materials and methods. A total of 10 random high-power ( $\times 40$ ) sections of each slide were then examined microscopically to determine the degree of leukocyte infiltration. Tumor infiltrate scores were assigned based on a scale of 0–4, where a score of 0 was equivalent to 0 infiltrating leukocytes, 1 was equivalent to 1–5 infiltrating cells, 2 was equivalent to 6–10 infiltrating cells, 3 was equivalent to 11–15 infiltrating cells, and 4 was equivalent to  $>15$  infiltrating cells, as described in Materials and methods. (a) Mean ( $\pm$  s.e.) infiltrate scores for pre- and post-treatment tumor biopsies were plotted for CD4+ T cells, CD8+ T cells, and macrophages. Statistical analysis of infiltrate scores did not reveal significant differences between pre- and post-treatment biopsies, although the difference in CD8+ T cell infiltrates approached significance ( $P=0.08$ ). (b, c) Marked infiltration of CD8+ T cells (red staining;  $\times 20$ ) into tumor tissues (c) was observed in one dog following six infusions of LDC encoding the endostatin gene, whereas pretreatment tumor biopsies (b) did not contain CD8+ T cells. Inset shows higher power view ( $\times 40$ ) of the T-cell infiltrate.

in a large animal spontaneous tumor model lead to transfection of cutaneous tumors. And secondly, could infusion of LDC inhibit tumor angiogenesis in large, well-established tumors. The answer to the first question appears to be no, in that we were unable to detect transgene expression in cutaneous tumor tissues following intravenous infusion of LDC. However, several caveats may apply. First, it may be that soft tissue sarcomas are more difficult to transfect than other tumor types, although it is likely that the major target for transfection by this route is probably the endothelium and not the tumor itself. Also, the LDC used for this study were optimized for pulmonary delivery of genes, and it may be that additional modifications of the LDC (e.g. addition of PEG groups to the liposome surface) might improve gene delivery to tumor tissues following intravenous delivery.



**Figure 6** Effects of LDC on endothelial cell survival and on release of tumor inhibitory cytokines by spleen cells. Studies were performed to assess gene nonspecific effects of LDC on endothelial cell survival and on release of tumor inhibitory cytokines. **(a)** Normal mouse capillary endothelial cells were plated in quadruplicate wells and were incubated with serially diluted LDC (formulated with noncoding plasmid DNA) in complete medium for 24 h, and then cell viability was determined by MTT assay, as described in Materials and methods. The mean ( $\pm$  s.d.) optical density reading for each dilution of LDC (expressed as DNA content) was plotted. Similar results were obtained in two additional experiments. **(b)** Mice ( $n=5$  per group) were injected intravenously with LDC (equivalent to  $10\ \mu\text{g}$  DNA per mouse), then the spleens were collected 6 h later and cultured for 18 h in complete medium and supernatants collected and frozen. Supernatants from spleens from untreated control mice were processed similarly. The supernatants were then diluted 1:1 and incubated with murine fibrosarcoma cells (MCA 2.1) in quadruplicate wells for 24 h, and then viability determined by MTT assay as described in Materials and methods. The mean optical density ( $\pm$  s.d.) for tumor cells treated with LDC-elicited or control spleen supernatants were then plotted. Similar results was obtained in one additional experiment (\* $P<0.01$ ).

More efficient tumor transfection might also be achieved if higher doses of the LDC were administered. However, doses much above the dose used in the current study ( $20\ \mu\text{g}$  DNA per kg body weight) would be likely to elicit toxicity, as was found in a previous study in dogs.<sup>42</sup>

Intravenous delivery of LDC appeared to inhibit tumor angiogenesis in some of the dogs in this study. The effect was, however, independent of the transgene delivered,

inasmuch as there were no significant differences between dogs treated with endostatin or luciferase DNA. Admittedly, with the small sample size evaluated in this study, it is impossible to exclude the possibility that endostatin gene delivery may have actually been more effective than infusion of an irrelevant plasmid. Nonetheless, our analysis suggested that any treatment effect from endostatin gene delivery was likely to be small. Also arguing against an endostatin specific effect was the fact that transgene expression in the tumors was not observed and that increased circulating concentrations of endostatin were not detected.

The antitumor effects observed in this study, therefore, appeared to be largely transgene independent and were most likely induced by the LDC themselves. There is ample precedent for noncoding LDC (empty vector) eliciting antitumor activity.<sup>36,38,50</sup> In addition, the ability of noncoding LDC to inhibit angiogenesis has also been demonstrated previously in mouse tumor models.<sup>21,24,51</sup> However, in the studies conducted in mice, the doses of LDC that were administered were much higher (up to 200 times more DNA per kg body weight) than the doses used in dogs in this study. Therefore, it is noteworthy that the effects observed in this study occurred at a very low dose of LDC, and in large animals with large, established spontaneous tumors. The slow infusion of LDC used for these studies was also an important difference between this study and studies typically conducted in mice. Large animals did not tolerate bolus infusions of LDC, and slow infusions also resulted in much more efficient gene delivery systemically (D Liggitt, unpublished data).

Although a placebo-treated control group was not included in this phase I study, the fact that eight animals experienced stabilization of their tumor growth and two had tumor regression suggests that a treatment effect was induced by repeated infusions of the LDC. For example, spontaneous regressions of soft tissue sarcomas in dogs are rare.<sup>39</sup> The effects of LDC infusion on tumor growth and tumor angiogenesis could have been mediated by a combination of factors, including direct endothelial cell injury by LDC, release of cytokines with antiangiogenic and antitumor properties, and recruitment of inflammatory cells into tumor tissues. For example, we observed that changes in tumor angiogenesis did not always correlate with tumor responses, as five of 10 dogs with stable disease or decreased tumor size also had a decrease in MVD, whereas MVD was unchanged or increased in the other five dogs. This finding suggested that a combination of antiangiogenic and direct antitumor effects was elicited by infusion of the LDC. For example, infiltration of CD8+ T cells was observed in tumor tissues of some dogs in this study following infusion of LDC. Thus, deposition of LDC in the tumor may have recruited CD8+ T cells into the tumor tissues, or triggered activation of local dendritic cells with subsequent stimulation of cytotoxic T cells, as has been reported previously.<sup>38</sup>

In summary, the studies reported here indicate that infusion of LDC appears to elicit substantial antitumor and antiangiogenic activity, probably mediated by the

immune stimulatory properties of the LDC. Further investigations of intravenous LDC infusion, alone or combined with radiation therapy or chemotherapy for tumor treatment, are warranted. For example, our preliminary observations suggest enhancement of tumor responses when dogs with advanced lung metastases were treated with LDC plus chemotherapy (R Elmslie, unpublished data). In addition, recent reports indicate that activation of innate immunity by administration of CpG oligonucleotides, which elicit immune responses very similar to those elicited by LDC, markedly enhances tumor sensitivity to radiation therapy.<sup>52-54</sup>

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