Effect of thalidomide on growth and metastasis of canine osteosarcoma cells after xenotransplantation in athymic mice

James P. Farese, DVM; Leslie E. Fox, DVM, MS; Carol J. Detrisac, DVM, PhD; James M. Van Gilder, BS; Sara L. Roberts; Jamie M. Baldwin, MStat

Objective—To determine whether thalidomide inhibits the growth of primary and pulmonary metastatic canine osteosarcoma in mice after xenotransplantation.

Animals—Athymic nude mice.

Procedure—Canine osteosarcoma cells were injected SC in 50 mice. Mice were randomly placed into the following groups: control group (n = 13; DMSO [drug vehicle] alone [0.1 mL/d, IP]); low-dose group (12; thalidomide [100 mg/kg, IP]), mid-dose group (13; thalidomide [200 mg/kg, IP]); and high-dose group (12; thalidomide [400 mg/kg, IP]). Starting on day 8, treatments were administered daily and tumor measurements were performed for 20 days. On day 28, mice were euthanatized and primary tumors were weighed. Lungs were examined histologically to determine the number of mice with metastasis and tumor emboli. Mean area of the pulmonary micrometastatic foci was determined for mice from each group.

Results—Primary tumor size and weight were not significantly different among groups. The number of mice in the mid-dose (200 mg/kg) and high-dose (400 mg/kg) groups with micrometastasis was significantly less than the number of control group mice; however, the number of mice with tumor emboli was not affected by thalidomide treatment. Size of micrometastasis lesions was not affected by thalidomide treatment.

Conclusions and Clinical Relevance—Mean area of micrometastases was not affected by treatment; however, growth of micrometastases had not yet reached an angiogenesis-dependent size. Although thalidomide did not affect growth of primary tumors in mice after xenotransplantation of canine osteosarcoma cells, our findings indicate that thalidomide may interfere with the ability of embolic tumor cells to complete the metastatic process within the lungs. (*Am J Vet Res* 2004;65:659–664)

Osteosarcoma is the most common primary bone neoplasia in dogs.[1] It is an aggressive tumor that metastasizes to the lungs by the time of diagnosis in nearly all dogs. Although much effort has been directed toward improving adjuvant treatment following surgical removal of appendicular osteosarcomas, most affected dogs eventually die from metastatic disease. Because micrometastatic tumors rely on neovascularization for growth beyond microscopic levels,[2] there has been considerable interest in evaluating drugs that have antiangiogenic properties.

Thalidomide is an immunomodulatory agent with anti-inflammatory activity.[3,4] In addition, thalidomide has antiangiogenic properties, the mechanism of which is unknown.[5-7] The use of thalidomide in the 1960s to treat the nausea associated with pregnancy resulted in an increased incidence of congenital dysmelia (stunted limb growth).[8] It has been suggested that the antiangiogenic effects of thalidomide may be responsible for the teratogenic effects on fetal limbs by inhibition of blood vessel growth in the developing fetal limb bud.[6,9] Thalidomide is presently used to effectively treat erythema nodosum,[10] graft-versus-host disease in transplant recipients,[11] aphthous ulceration in patients with human immunodeficiency virus,[12] Crohn's disease,[13] and multiple myeloma.[14-16]

Rodents with induced neoplasias are commonly used to evaluate chemicals for chemotherapeutic effects and chemoprophylaxis. Induced neoplasias in rodents have been used for study on the basis of the reproducibility of the neoplasia, low cost, and time effectiveness. Thalidomide has been evaluated for use in the treatment of experimentally induced tumors in mice,[17,18] with results varying from no apparent antitumor effect to substantial inhibition of primary tumor growth, primary tumor microvessel density, and pulmonary metastasis.[17,18] Efficacy of antiangiogenic treatment in mice for allografted osteosarcoma has been evaluated for the angiogenesis inhibitor TNP-470.[19] In that report, TNP-470 decreased the extent of pulmonary metastatic disease in a dose-dependent manner. Although some studies[20,21] have characterized canine osteosarcoma cell lines after xenotransplantation in mice, none have been used to evaluate the

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From the Departments of Small Animal Clinical Sciences (Farese, Van Gilder, Roberts) and Pathobiology (Detrisac), College of Veterinary Medicine, and the Department of Statistics, Institute of Food and Agricultural Sciences (Baldwin), University of Florida, Gainesville, FL 32610; and the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011 (Fox).
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Address correspondence to Dr. Farese.

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antimetastatic effects of antiangiogenic drugs on canine osteosarcoma cells. Barroga et al[22] developed the **highly metastasizing parent osteosarcoma (HMPOS)** cell line from a parent cell line (ie, POS) that originated from a spontaneously occurring proximal femoral osteosarcoma in a 1.5-year-old dog.[23] When athymic mice are inoculated SC with canine HMPOS cells, primary tumor development is visible approximately 1 week after inoculation and pulmonary metastasis is evident histologically as early as 4 weeks after inoculation.

The purposes of the study reported here were to determine whether the study of canine HMPOS cells after xenotransplantation in mice provides a useful means to evaluate efficacy of antiangiogenic drugs, determine whether thalidomide inhibits the growth of primary tumors induced by canine HMPOS cells, determine whether thalidomide inhibits the development of pulmonary metastasis, and determine whether the effect of thalidomide is dose dependent. Because gross metastatic disease limits survival of dogs with appendicular osteosarcoma, the effect of this drug on metastasis was considered the most clinically relevant objective of this study. Because of its teratogenic effects, the FDA strictly regulates thalidomide. If thalidomide proved to be effective in the inhibition of canine HMPOS cells after xenotransplantation in mice, use of thalidomide to treat micrometastatic disease in dogs with appendicular osteosarcoma could be justified.

Materials and Methods

Cell line—The canine HMPOS cells[a] were seeded into 150-cm^2 flasks in RPMI-1640-containing, 10% charcoal-stripped, and ultraviolet-light treated fetal calf serum; L-glutamine; and antibiotics (penicillin [0.0625 g/L] and streptomycin [0.1 g/L]) at 37°C under 5% CO_2 and 95% room air. Cells were grown to confluence, detached from the plates with 0.25% trypsin, washed with PBS solution (pH, 7.4), and counted with a hemacytometer. Cells were resuspended to a concentration of 5×10^6 cells/0.25 mL with > 90% viability, as determined by a trypan blue dye exclusion assay, for SC inoculation.

Animals—Fifty 5-week-old female athymic nude mice of strain Hsd[b] were housed in specific pathogen-free conditions at the University of Florida, Health Science Center. The Institutional Animal Care and Use Committee approved the study protocol. Fifty mice were randomly assigned to 1 of 4 groups (12 or 13 mice/group). All mice received 5×10^6 canine HMPOS cells SC between the scapulae on day 0.

Preparation and thalidomide administration—Thalidomide[c] was dissolved in **dimethyl sulfoxide (DMSO)** to concentrations of 25, 50, and 100 mg/mL. The 3 drug solutions were formulated to allow all mice to receive approximately 0.1 mL of the drug solution/d. Each drug solution was passed through a filter with a pore size of 20 µm. Mice were grouped as follows: control group mice (n = 13; DMSO [drug vehicle] alone [0.1 mL/d, IP]), low-dose group mice (12; thalidomide [100 mg/kg, IP]), mid-dose group mice (13; thalidomide [200 mg/kg, IP]),[17,18] and high-dose group mice (12; thalidomide [400 mg/kg, IP]). In all mice, IP injections were given by use of a 25-gauge needle starting 8 days after tumor cell inoculation and then given daily for 20 days.

Under specific pathogen-free conditions, mice were weighed every other day and observed daily for primary tumor growth at the site of inoculation, changes in behavior, and general appearance. Following gross appearance of the tumor, the tumors were also examined daily for changes, such as ulceration in the overlying skin. Starting on day 8, tumor size was measured with calipers (mm) every other day. The longest dimension of the tumor was considered the length, and the distance perpendicular to the length was considered the width. Tumor size was then calculated by use of a previously reported formula[22] as follows: tumor size (mm) = $\sqrt{(\text{length} \times \text{width})}$. All mice were euthanatized on day 28 by IP injection of sodium pentobarbital (150 mg/kg).

Necropsy and histologic examination—Mice were reweighed after euthanasia. If a primary tumor was present between the scapulae, it was dissected free and weighed. The primary tumor was then fixed in neutral-buffered 10% formalin. A complete necropsy was performed, and the respiratory tract (larynx to lung), heart, and all mediastinal adipose tissue were removed and weighed. The lungs were then inflated with neutral-buffered 10% formalin. After fixing in formalin for 24 hours, all tissues were then transferred into 70% alcohol.

The large left lung lobe was bisected in a cranial to caudal direction. The 4 right lung lobes were removed from the right bronchus and bisected similarly. This yielded 1 section from each of the 5 lung lobes and a total of 5 sections/mouse. A 5-µm-thick section of the primary tumor and of each of the lung lobes from each mouse was embedded in paraffin and stained with H&E for microscopic examination. Lung sections were examined to determine the absence or presence of pulmonary metastatic lesions and tumor emboli in the pulmonary vasculature. Tumor foci were considered a metastasis lesion when all or a portion of the aggregate of cells was located in the pulmonary parenchyma and an embolus when the entire aggregate of cells was contained within a blood vessel (**Fig 1**). The pathologist was blinded to experimental treatment.

Area of the individual metastatic foci—The cross-sectional area (as represented by the number of computer pixels) of each micrometastatic focus was determined for each mouse that developed pulmonary micrometastatic disease. This was accomplished by imaging each of the metastatic foci with a digital camera[d] at a standard focal distance through the eyepiece of a standard light microscope by use of the 10× objective. The resulting digital images were then imported to a computer. By use of image software,[e] each of the metastatic foci was then traced and the resulting area in pixels was recorded.

Data analysis—A spreadsheet was created by use of commercially available software,[f] and all statistical analyses were performed by use of computer software.[g] Any relevant assumptions, such as normality of the error terms, were formally verified, and significance was set at a value of $P < 0.05$. Mice that did not have a detectable primary tumor present by day 28 and did not have evidence of metastatic disease on histologic examination were not considered in the final data analysis. Mice that died or were euthanatized prior to the end of the study were included in the analysis. An ANCOVA, by use of generalized least squares estimation, was used to compare primary tumor weight at necropsy. Because pulmonary metastasis and pulmonary tumor emboli are binary (positive [present] or negative [absent]) findings, odds of pulmonary metastasis or pulmonary tumor emboli was analyzed by logistic regression. Explanatory variables for the regressions included body weight, primary tumor weight at necropsy, and treatment.

To evaluate the potential inhibitory effects of thalidomide on the growth of micrometastasis lesions, mean area of the individual metastatic foci was compared among groups by use of mixed-model methods and an ANCOVA. This was done while taking into account the weight of the primary

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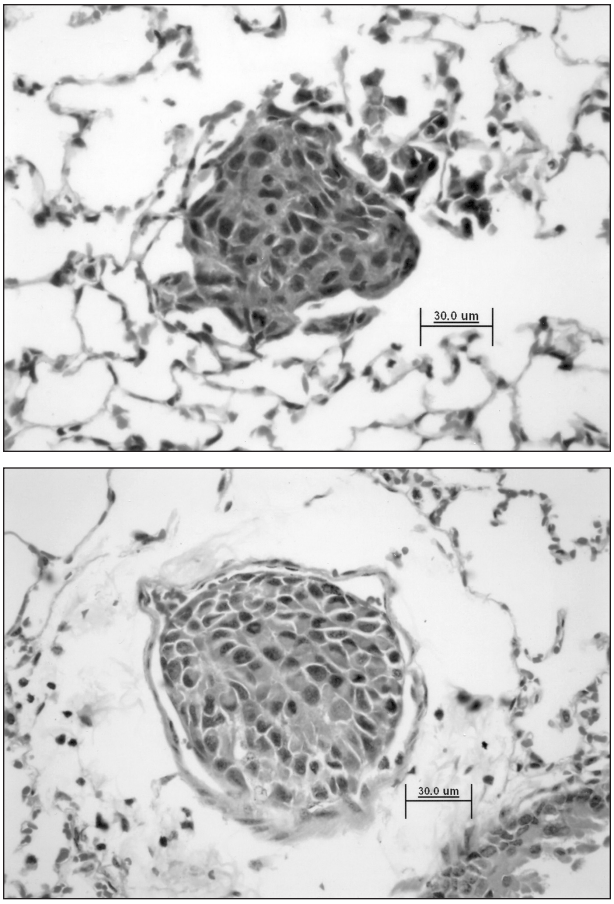

Figure 1—Photomicrographs of sections of lungs from athymic mice. Top panel—Pulmonary metastatic canine osteosarcoma in a mouse after xenotransplantation. Bottom panel—Pulmonary embolic canine osteosarcoma in a mouse after xenotransplantation. Notice that the tumor embolus is contained completely within the pulmonary vessel. Several erythrocytes are visible in the space between the outer surface of the tumor embolus and the surrounding vessel wall. H&E stain; bar = 30 μm.

tumor, body size, and correlation between observations within each mouse (metastasis sizes within a mouse are expected to be correlated).

Results

At the time of treatment initiation (ie, 8 days after inoculation), 40 of the 50 mice had a detectable primary tumor present at the injection site. At the termination of the study, 48 of the 50 mice had developed a primary tumor. All of these 48 mice were included in the final analysis. The 2 mice not considered in the final analysis as a result of lack of primary tumor growth were both in the control group. Five (1 from the control group, 2 from the low-dose [100 mg/kg] group, and 2 from the mid-dose [200 mg/kg] group) mice either died or were euthanized prior to the end of the study as a result of physical deterioration. All 5 of these mice developed a primary tumor, and 4 of the 5 had evidence of pulmonary metastasis. Because these 5 mice died toward the latter part of the study, each was included in the final analysis.

Mean tumor size in mice of the thalidomide-treatment groups was not significantly different from that of mice in the control group on any day (**Fig 2**). When

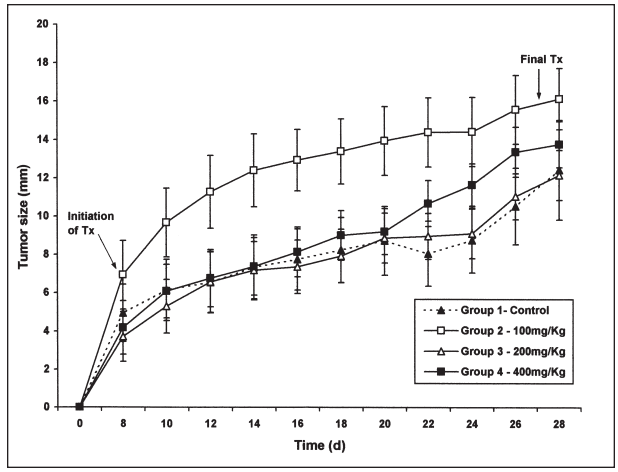

Figure 2—Mean (± SD) primary tumor size (mm^2) versus time (d). Treatment (Tx) with either the drug vehicle DMSO (control; n = 11 mice) alone or thalidomide (100 [12], 200 [13], and 400 [12] mg/kg]) dissolved in dimethyl sulfoxide (DMSO) was initiated on day 8 and continued daily until day 28. Tumor measurements were made every other day from days 8 to 28. Differences among groups were not significant on any day.

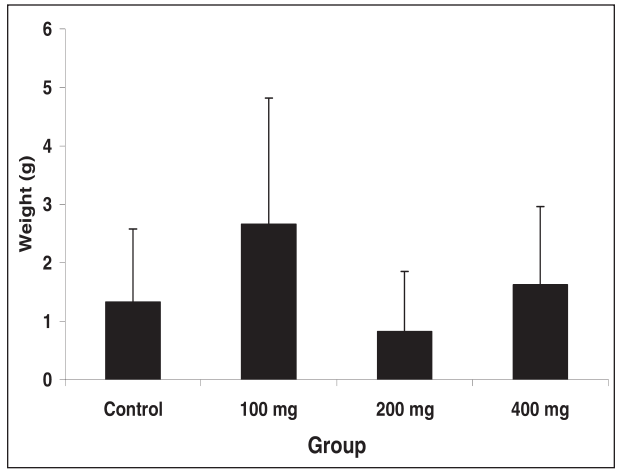

Figure 3—Mean (± SD) primary tumor weight (g) at necropsy among the following groups: control group (drug vehicle [DMSO] alone [n = 11 mice]) or thalidomide-treatment groups (100 [12], 200 [13], and 400 [12] mg/kg). Tumors were carefully dissected free from the skin and subcutaneous tissues and weighed. Differences among groups were not significant.

mean primary tumor weight obtained at necropsy was compared, no significant difference was found among groups (**Fig 3**). The number of mice with pulmonary metastasis was significantly lower in the mid-dose (200 mg/kg) and high-dose (400 mg/kg) groups (P = 0.03 and 0.02, respectively), compared with control group mice (**Fig 4**). Holding all variables constant, the odds of metastasis in control group mice was 19 (95% confidence interval, 1.26 to 303.03) and 33 (95% confidence interval, 1.81 to 588.23) times as likely as in the mid-dose (200 mg/kg) and high-dose (400 mg/kg) group mice, respectively. The number of mice with pulmonary metastasis in the low-dose (100 mg/kg) group was not significantly different from that of the control group. The number of mice with tumor emboli was not affected by treatment. Differences in mean cross-sectional area of the metastatic lesions among groups were not significant (**Fig 5**).

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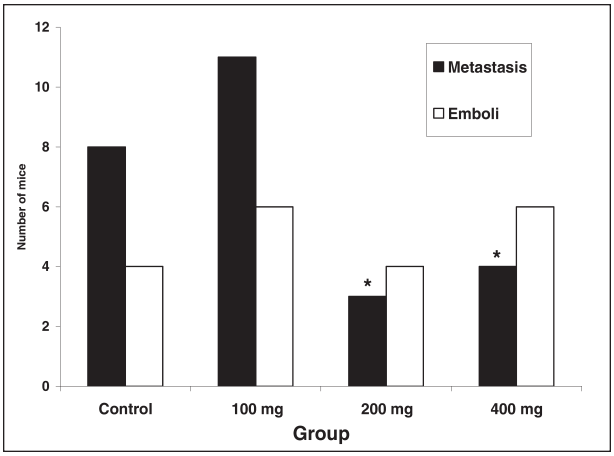

Figure 4—Number of mice within the control group (drug vehicle [DMSO] alone [n = 11 mice]) or thalidomide-treatment groups (100 [12], 200 [13], and 400 [12] mg/kg) that had pulmonary metastasis or tumor emboli. *Significant ($P < 0.05$) difference between the thalidomide-treatment groups and control group.

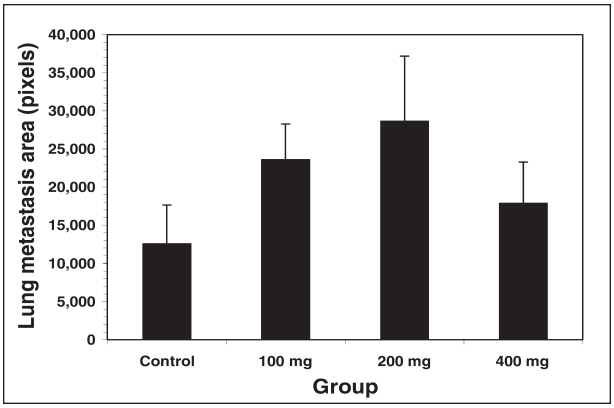

Figure 5—Mean (± SD) cross-sectional area of pulmonary metastatic foci (pixels) among the following groups: control group (drug vehicle [DMSO] alone [n = 11 mice]) or thalidomide-treatment groups (100 [12], 200 [13], and 400 [12] mg/kg). Differences among groups were not significant.

Necropsy and subsequent histologic examination of tissues revealed adhesions and inflammation in the peritoneal cavity and spongiosis lesions in the brain, most often in the mesencephalon. These findings were present in both the control group mice and mice in the thalidomide-treatment groups.

Discussion

Thalidomide has been reported to have the potential to inhibit development of metastatic disease.[17,18] Results of our study reveal a dose-dependent inhibitory effect of thalidomide on the development of pulmonary metastasis of canine osteosarcoma cells in mice after xenotransplantation. A significant decrease in the number of mice with pulmonary metastatic disease was found with dosages of thalidomide of 200 and 400 mg/kg/d but not with a dosage of 100 mg/kg/d. These results are consistent with those of previous studies[17,18] in which IP administration of thalidomide at a dosage of 200 mg/kg/d was effective in the inhibition of pulmonary metastasis. In our study, however, thalidomide failed to have an inhibitory effect on growth of primary tumors induced with canine HMPOS cells.

In a study that investigated the effects of thalidomide on Lewis lung carcinoma cells implanted SC into nude mice, thalidomide (200 mg/kg/d, IP) had no effect on primary tumor growth but decreased the incidence of pulmonary metastatic disease.[18] Interestingly, although the occurrence of pulmonary micrometastasis was significantly decreased by thalidomide treatment in our study, the development of tumor emboli was not. As tumor emboli have not completed the metastatic process by exiting the vasculature, we opted to distinguish these intravascular neoplastic aggregates from those that were at least partially located in the lung parenchyma. Our findings suggest that thalidomide did not prevent cells from leaving the primary tumor and gaining access to systemic circulation but may have interfered with the ability of the embolic cells to complete the metastatic process through the vessel wall into the lung parenchyma. Thalidomide may have interfered with attachment of tumor cells to the endothelial cell surface, a process known to occur prior to migration of tumor cells through the vessel wall. Results of several studies[24-26] indicate that thalidomide alters the density of important cellular adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1; the anti-inflammatory effect of thalidomide has been attributed, at least in part, to inhibition of leukocyte-endothelium interaction.[27,28]

In our study, mean area of the micrometastatic foci did not significantly differ among groups. The small size of the micrometastasis lesions at the time of euthanasia (< 1 mm in diameter) is important. Since tumors can grow up to 2mm in diameter without developing a blood supply, metastatic tumor growth may not have reached an angiogenesis-dependent stage.[2] Thus, it is not surprising that antiangiogenic treatment with a drug such as thalidomide would have no effect on micrometastatic tumor growth. Evaluation of canine osteosarcoma cells after xenotransplantation in mice that have been treated with thalidomide during a time when metastatic tumors are dependent on angiogenesis would help to determine whether the antiangiogenic effects of thalidomide could inhibit neovascularization of pulmonary micrometastatic lesions. Surgical removal of the primary tumor (once pulmonary metastasis has occurred) could be performed to allow more time for micrometastatic tumor growth without subjecting mice to inhumane conditions and to more closely simulate the clinical treatment of canine appendicular osteosarcoma.

It is possible that an effect on primary tumor growth may have been found if mice were treated for a longer time. In the study by Kotoh et al,[17] a significant decrease in primary tumor size and microvessel density was not seen until day 21 of the 35 days of treatment with thalidomide (200 mg/kg/d, IP). Many mice in our study had large tumors by day 20 of treatment that were incompatible with humane animal care and clinical relevance. It is possible that thalidomide might have been effective in inhibiting primary tumor growth if the tumors had been smaller at the time treatment was initiated, as tumor burden would have been less

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and longer treatment duration would have been possible. To achieve this, fewer cells could have been used in the inoculum or treatment could have been started earlier in the course of tumor development. Delaying treatment until day 8 was opted to more closely mimic the clinical scenario, in which primary tumors are well established prior to treatment. It is also possible that an inhibitory effect of thalidomide on primary tumor growth might have been found if more canine osteosarcoma cell lines had been evaluated. In a study that evaluated the effect of thalidomide on 2 human esophageal squamous cell carcinoma cell lines,[17] the drug was only effective against tumors from 1 of the cell lines. Thus, before conclusions can be made about the effect of thalidomide on the growth of primary canine osteosarcomas, evaluation of other cell lines should be performed.

Necropsy and subsequent histologic examination of tissues revealed spongiosis lesions in the brain, most often in the mesencephalon. Because this was found in control group mice and mice of the thalidomide-treatment groups, the vehicle DMSO may have caused these lesions. Toxicologic studies in humans and animals report numerous adverse effects from DMSO administration, including CNS effects, and there is a report[29] of leukoencephalopathy in a human patient associated with reinfusion of DMSO-preserved stem cells. At this time, the consequence of the spongiosis lesions in our study is unknown. We selected DMSO as the drug vehicle because thalidomide has extremely low solubility in water and saline (0.9% NaCl) solution but does dissolve readily in DMSO. Complete dissolution allows the solution to be sterile-filtered without affecting the drug concentration during the filtering process.

A limitation of this study was that medical treatment of primary osteosarcoma is not the typical form of treatment; rather, some form of tumor removal (with or without adjunctive chemotherapy) is usually performed. However, as radiation therapy is increasingly being used for treatment of appendicular osteosarcoma,[30-33] some dogs with limb-sparing surgery develop recurrence of the primary tumor,[1] and because pet owners are interested in alternative medical options, it is important to determine whether thalidomide is effective in controlling primary tumor growth.

In our study, canine osteosarcoma cells easily underwent xenotransplantation to mice and pulmonary metastasis occurred rapidly. The ability of thalidomide to lower the occurrence of pulmonary metastasis in the mice of our study is an interesting finding. Additional studies need to be performed to investigate the effect of thalidomide on interactions between tumor cells and vascular endothelium and determine the effect of thalidomide on canine osteosarcoma micrometastatic tumor growth in mice. Surgical removal of the primary tumor should be considered in future studies evaluating antiangiogenic agents for inhibition of growth of micrometastases induced with the HMPOS cell line in mice.

[a]HMPOS cells provided by Dr. Tsuyoshi Kadosawa, Hokkaido University, Sapporo, Japan.
[b]Athymic Nude-*nu* mice, Harlan, Indianapolis, Ind.
[c]Pediatric Pharmaceuticals, Iselin, NJ.
[d]CoolPix 990, Nikon, Melville, NY.
[e]Sigma Scan, SPSS Science, Chicago, Ill.
[f]Excel, Microsoft Corp, Redmond, Wash.
[g]SAS/STAT, version 6.12 for Windows, SAS Institute Inc, Cary, NC.

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