Effect of bevacizumab on angiogenesis and growth of canine osteosarcoma cells xenografted in athymic mice

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Objective—To investigate the effects of bevacizumab, a human monoclonal antibody against vascular endothelial growth factor, on the angiogenesis and growth of canine osteosarcoma cells xenografted in mice.

Animals—27 athymic nude mice.

Procedures—To each mouse, highly metastasizing parent osteosarcoma cells of canine origin were injected into the left gastrocnemius muscle. Each mouse was then randomly allocated to 1 of 3 treatment groups: high-dose bevacizumab (4 mg/kg, IP), low-dose bevacizumab (2 mg/kg, IP), or control (no treatment). Tumor growth (the number of days required for the tumor to grow from 8 to 13 mm), vasculature, histomorphology, necrosis, and pulmonary metastasis were evaluated.

Results—Mice in the high-dose bevacizumab group had significantly delayed tumor growth (mean ± SD, 13.4 ± 3.8 days; range, 9 to 21 days), compared with that for mice in the low-dose bevacizumab group (mean ± SD, 9.4 ± 1.5 days; range, 7 to 11 days) or control group (mean ± SD, 7.2 ± 1.5 days; range, 4 to 9 days). Mice in the low-dose bevacizumab group also had significantly delayed tumor growth, compared with that for mice in the control group.

Conclusions and Clinical Relevance—Results indicated that bevacizumab inhibited growth of canine osteosarcoma cells xenografted in mice, which suggested that vascular endothelial growth factor inhibitors may be clinically useful for the treatment of osteosarcoma in dogs.

Impact for Human Medicine—Canine osteosarcoma is used as a research model for human osteosarcoma; therefore, bevacizumab may be clinically beneficial for the treatment of osteosarcoma in humans. (Am J Vet Res 2013;74:771–778)
That treatment protocol results in a 1-year survival rate of 50% and a 2-year survival rate of 20%. The survival rate in human patients with osteosarcoma is somewhat higher (3-year survival rate, 60%) following surgery and chemotherapy. However, the overall survival rates for both veterinary and human patients with osteosarcoma have remained fairly static over time despite the use of aggressive surgical and chemotherapy protocols; this fact suggests the need for the development of adjuvant treatments to enhance current treatment protocols.

Because the growth of many tumors is dependent on an abundant blood supply, the use of antiangiogenic agents for the treatment of various types of tumors has garnered the interest of both veterinary and human oncologists. Two general strategies have been developed for the inhibition of tumor vasculature: treatments that disrupt the angiogenesis of tumor vasculature and those that compromise the established vasculature of solid tumors. Most of the research regarding antiangiogenesis has focused on VEGF because it is one of the most potent stimulators of angiogenesis. Results of 1 study indicate that amplification of the VEGF pathway within osteosarcoma tissue specimens obtained from human patients is negatively associated with tumor-free survival time. Thus, several drugs that inhibit VEGF activity have been developed, and the use of VEGF inhibitors reduces tumor volume and suppresses the metastatic potential of xenografted osteosarcoma cells.

Bevacizumab is a commercially available antiangiogenic agent that consists of a human monoclonal antibody against VEGF. The FDA approved bevacizumab in 2004 for use as part of the primary treatment of metastatic colorectal cancer in human patients. Since then, the use of bevacizumab has had beneficial effects in the treatment of various carcinomas and sarcomas in human patients. In a study that involved human patients with metastatic soft tissue sarcomas, the use of bevacizumab in combination with doxorubicin resulted in improved disease stabilization in the majority of patients, compared with disease stabilization in patients treated with doxorubicin alone. Presently, a clinical trial is underway to investigate the safety and potential efficacy of bevacizumab as an adjunctive treatment for osteosarcoma in human patients. To our knowledge, reports on the efficacy of bevacizumab for the treatment of osteosarcoma, regardless of species, are lacking.

The purpose of the study reported here was to evaluate the effects of bevacizumab on the angiogenesis and growth of canine osteosarcoma cells xenografted in athymic mice. Although long-term use of a human monoclonal antibody such as bevacizumab in dogs would likely be unfeasible, the determination of whether bevacizumab causes a reduction in tumor growth is important for the elucidation of the role of angiogenesis inhibition in the treatment of osteosarcoma in dogs. Given the similarities of osteosarcoma development in dogs and humans, results of this study may also be beneficial for investigators researching the use of bevacizumab as an adjunctive treatment for osteosarcoma in human patients.

Materials and Methods

Animals—Twenty-seven 5-week-old nude athymic mice were used for the study. The mice were housed in a specific pathogen-free barrier facility, exposed to 12-hour light and dark cycles, and provided ad libitum access to sterilized food and water. Throughout the duration of the study, each mouse was weighed every other day and observed daily for changes in behavior and general appearance. All study protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida, and all experiments were conducted at that university.

Study design—To each mouse, HMPOS cells of canine origin were injected into the left gastrocnemius muscle. Each mouse was then randomly allocated to 1 of 3 treatment groups: high-dose bevacizumab (4 mg/kg, IP, twice weekly on Monday and Friday), low-dose bevacizumab (2 mg/kg, IP, twice weekly on Monday and Friday), or an untreated control group. The assigned treatment was initiated for each mouse when the maximum diameter of the left hind limb was 8 mm (equivalent to a tumor mass of 0.2 g) and continued for 2 cycles (ie, 4 treatments) or until the maximum diameter of the left hind limb was 13 mm and the mouse was euthanized. Immediately following euthanasia, a necropsy was performed on each mouse, and specimens of the primary tumor and lungs were histologically examined.

HMPOS cell culture—The HMPOS cells used in the study were a pulmonary metastatic subtype of a parent osteosarcoma, which originated from the proximal aspect of a femur of a 1.5-year-old male dog. The HMPOS cells were propagated in culture as described to create the suspension that was injected into the mice. Briefly, the culture media used for HMPOS cell propagation consisted of RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin-streptomycin (penicillin, 10,000 U/mL; streptomycin, 10,000 µg/mL), 1% l-glutamine, 2% vitamin solution, and 1% nonessential amino acids. Cells (2 × 10⁵) were seeded into 150-cm² flasks and maintained at 37°C under 5% CO₂ and 95% room air until they reached confluence (passage time, 3 days). The HMPOS cells were then washed with Hanks solution (pH, 7.4), detached from the flask with 0.25% trypsin, resuspended in culture media, and counted with a hemocytometer. Subsequently, the cells were precipitated and resuspended in PBS solution (pH, 7.4) to a concentration of 5 × 10⁶ cells/0.02 mL (25 × 10⁶ cells/mL) for IM inoculation. This suspension was transported on ice to the animal housing facility.

Determination of VEGF secretion—A human VEGF immunoassay was used to determine the amount of VEGF secreted by the HMPOS cells. Briefly, 1.5 × 10⁶ HMPOS cells were seeded onto a 60-mm plate, sufficient HMPOS culture media was added to the plate to create a volume of 5 mL, and the plate was incubated at 37°C under 5% CO₂ and 95% room air until the cells reached approximately 70% confluence (10 days). The culture media was aspirated from the plate, discarded, and replaced with 2 mL of fresh culture media. The
plate was incubated for an additional 24 hours, after which the culture media was collected and centrifuged at 200 × g and 4°C for 10 minutes. The cells were trypsinized and counted with a hemocytometer, and the top 1 mL of media from each sample was removed and immediately analyzed via the immunoassay. Secretion of VEGF was determined for 3 samples of HMPOS cells, each of which was assayed in duplicate.

**Tumor induction and measurement**—Into the left gastrocnemius muscle of each mouse, 5 × 10⁵ HMPOS cells suspended in 0.02 mL of PBS solution were injected. The mice were examined daily for evidence of a primary tumor at the injection site. Following the initial appearance of the tumor in each mouse, the tumor-bearing limb was measured and examined daily for morphological changes such as skin ulceration. Maximum tumor diameter was determined with a metric circle template as described. Each tumor diameter measurement was then converted to tumor volume via the following formula: tumor volume = (1/6)πd³ – 100, where d is the diameter of the tumor-bearing leg at the level of the tumor and 100 represents a volume correction factor for a mouse leg without a tumor. Tumor weight was approximated from the tumor volume via the estimation that 100 mm³ is equivalent to approximately 0.1 g, and the tumor percentage of body weight for each mouse was monitored in accordance with University of Florida institutional guidelines.

**Necropsy and histologic examination**—Each mouse was euthanized via CO₂ with thoracotomy confirmation when the left hind limb reached a maximum diameter of 13 mm at the level of the tumor (tumor weight, approx 1.0 g). Immediately after euthanasia, a complete necropsy was performed. Specimens of lung tissue were fixed in 10% neutral-buffered formalin and then embedded in paraffin. The primary tumor was dissected with the limb in situ, bisected at its greatest diameter, fixed in neutral-buffered 10% formalin for 48 hours, transferred to 70% alcohol, and then embedded in paraffin. Paraffin-embedded lung tissue and tumor specimens were cut into sections 5 μm thick, deparaffinized, and stained with H&E stain for histologic examination.

Lung tissue specimens were evaluated for the presence of metastases. Tumor specimens were evaluated for cell morphology and extent of necrosis. To determine extent of tumor necrosis, a digital image of the tumor at its maximum diameter was obtained via a digital camera attached to a microscope. That image was then processed with software and analyzed with an image-processing and -analysis program. The percentage of tumor necrosis was calculated as described: (necrotic tumor area/total tumor area) × 100. All histologic examinations and calculations of percentage of tumor necrosis were performed by 1 board-certified veterinary pathologist (DPT) who was unaware of the treatment assigned to each mouse.

**Determination of tumor angiogenesis**—An immunohistochemical staining protocol as described was used to quantitate angiogenesis within each tumor. Briefly, each slide that contained a 5-μm-thick section of tumor tissue was deparaffinized in xylene, rinsed in Tris buffer, and incubated in 3% H₂O₂ for 10 minutes to stop endogenous peroxidase activity. Heat-induced epitope retrieval was performed for 20 minutes, and the slides were cooled and rinsed with distilled water. The primary antibody CD31 (1:100 dilution) was then applied to each slide, followed by streptavidin peroxidase reagent and chromogen 3,3′-diaminobenzidine. The slides were then counterstained with hematoxylin, acetic acid reagent, and bluing reagent. Specimens of human tonsil tissue and mouse heart and kidney tissues were used as positive controls.

To evaluate angiogenesis, the stained slides were scanned with a microscope under low power (40X and 100X) to identify the areas of the tumor that had the highest concentrations of microvessels that stained positive for CD31, which represented areas of neovascularization. Within each of those areas, the number of individual vessels in one 200X field were counted. For each tumor section, the extent of angiogenesis was defined as the highest number of microvessels identified in any 200X field evaluated. Angiogenesis was determined for each tumor by 1 board-certified veterinary pathologist (DPT) who was unaware of the treatment assigned to each mouse.

**Data analysis**—The effect of treatment on the primary tumor was evaluated by rate of tumor growth, which was defined as the number of days required for the tumor-bearing limb to grow from 8 to 13 mm. A 1-way ANOVA with a Tukey adjustment for multiple comparisons was used to compare differences among the treatment groups for the following respective outcomes: rate of tumor growth, percentage tumor necrosis, microvessel density, and body weight at euthanasia. A mixed-effects linear regression model was used to compare daily tumor growth rates among the treatment groups. Kaplan-Meier survival analysis was used to evaluate time to euthanasia (ie, time from initiation of treatment [tumor-bearing limb diameter, 8 mm] until the tumor-bearing limb reached a diameter of 13 mm), and a log-rank test was used to compare differences in mean survival time among the treatment groups. All analyses were performed with statistical and graphing software, and values of P < 0.05 were considered significant.

**Results**

**Animals**—None of the mice developed any signs of bevacizumab toxicosis (ie, bleeding) during the study. During the early stages of the observation period, no changes in behavior or general appearance were detected for any of the mice aside from the development of a tumor in the left hind limb. During the latter stages of the observation period (ie, just prior to euthanasia), each mouse developed a slight mechanical impairment of the left hind limb that was attributed to the advancing size of the tumor. Tumor ulceration and swelling of the left hind limb distal to the tumor were not observed in any of the mice.
Mean ± SD body weight at euthanasia was 17.4 ± 0.8 g for mice in the control group, whereas the mean body weights for mice in the low-dose and high-dose bevacizumab groups were 18.6 ± 1.1 g and 18.3 ± 1.2 g, respectively. The mean body weight at euthanasia for the mice in the low-dose bevacizumab group was significantly (P = 0.041) greater than that for the mice in the control group.

Mean ± SD survival times were 13.4 ± 3.8 days, 9.4 ± 1.5 days, and 7.2 ± 1.5 days for mice in the high-dose bevacizumab, low-dose bevacizumab, and control groups, respectively (Figure 1). The mean survival time for mice in the high-dose bevacizumab group was significantly (P < 0.001) longer, compared with the mean survival times for mice in the low-dose bevacizumab and control groups. Although the mean survival time for mice in the low-dose bevacizumab group was longer than that for mice in the control group, the difference was not significant.

VEGF secretion by HMPOS cells—
The amount of VEGF secreted by HMPOS cells ranged from 1,210 to 1,600 pg/10^6 cells/d. Mean ± SD VEGF secretion by HMPOS cells was 1,420 ± 194 pg/10^6 cells/d.

Necropsy results—No gross abnormalities aside from a primary tumor on the left hind limb were detected in any of the mice during necropsy. Also, no micrometastases were identified during histologic evaluation of any of the lung tissue specimens.

Tumor growth and morphology—
Mean daily tumor size from treatment initiation (tumor size, 8 mm) until mice were euthanized (tumor size, 13 mm) was plotted for each treatment group (Figure 2). Results of the mixed-effects linear regression analysis indicated that the tumor growth rate for mice in the high-dose bevacizumab group was significantly (P < 0.001) slower than the tumor growth rates for mice in the low-dose bevacizumab and control groups. Also, the tumor growth rate for the mice in the low-dose bevacizumab group was significantly (P < 0.001) slower than the tumor growth rate for the mice in the control group.

Histologically, all tumors were described as having an infiltrative growth pattern. Tumor cells were either round or polygonal, with a moderate amount of cytoplasm and round-to-oval, chromatin-stippled nuclei, and no tumor cells infiltrated the tumor vasculature. All tumors contained both osteoid and boney tissues. The mean ± SD percentage of tumor necrosis was 7.5% ± 8.4% for mice in the high-dose bevacizumab group, 13.1% ± 12.2% for mice in the low-dose bevacizumab group, and 4.2% ± 3.1% for mice in the control group; the mean percentage of tumor necrosis did not differ significantly among the treatment groups.
The mean ± SD microvessel density within the tumors was 53.1 ± 20.7 vessels/200× field for mice in the high-dose bevacizumab group, 68.4 ± 28.8 vessels/200× field for mice in the low-dose bevacizumab group, and 150.3 ± 31.2 vessels/200× field for mice in the control group. The mean microvessel density within the tumors of mice in both the low-dose and high-dose bevacizumab groups was significantly (P < 0.001) lower, compared with that within the tumors of mice in the control group (Figure 3). The mean microvessel density within the tumors of the mice in the low-dose bevacizumab group did not differ significantly, compared with that within the tumors of the mice in the high-dose bevacizumab group.

Discussion

Results of the present study indicated that treatment with bevacizumab slowed the growth of canine osteosarcoma cells that were xenografted in athymic mice in a dose-dependent manner. Bevacizumab is a human monoclonal antibody that binds and inhibits VEGF. Vascular endothelial growth factor is necessary for angiogenesis within a tumor; thus, inhibition of VEGF slows vascularization and growth of a tumor. Bevacizumab has been approved by the FDA for use in combination with 5-fluorouracil–based chemotherapy agents for the treatment of metastatic colorectal cancer in human patients22,35,36 and has been used in combination with various other chemotherapy agents for the treatment of other types of cancer in human patients.35,36 To our knowledge, the present study was the first to find an antitumor effect of bevacizumab on osteosarcoma in any species.

In the present study, tumor growth in mice of the high-dose bevacizumab group was significantly slower than that in mice of the low-dose bevacizumab or control groups. Although tumor growth for mice in the high-dose bevacizumab group was similar to that for mice in the low-dose bevacizumab and control groups during the first week of treatment, it plateaued during the second week of treatment, which suggested that it required approximately 1 week for the effects of bevacizumab to become clinically evident. The dosing frequency for bevacizumab used in the present study was chosen on the basis of that of another study37 in which bevacizumab was used to treat solid tumors of human origin that had been xenografted in nude mice. For the present study, bevacizumab was administered twice weekly to increase the likelihood for detection of a treatment effect in the tumor model used. The control mice of the present study did not receive a placebo injection; thus, it is possible that the changes detected in the mice treated with bevacizumab were the result of the physical injection rather than an actual therapeutic effect of the drug. This seems unlikely, however, because results of other studies38,39 indicate that injection of a placebo of equal volume and by the same route as a VEGF inhibitor had no effect on tumor growth. The decreased microvessel density in the tumors of the mice that were treated with bevacizumab, compared with that in control mice, suggested that the observed inhibition of tumor growth was the result of the antiangiogenic effects of bevacizumab caused by inhibition of VEGF. Furthermore, the results of the present study suggested a positive association between tumor growth inhibition and dose of bevacizumab administered.

That bevacizumab treatment was associated with decreased microvessel density but not increased tumor necrosis was somewhat surprising but not totally unexpected. A decrease in microvessel density could result in a lack of oxygen supply to some areas of the tumor and cause hypoxia, but the extent of hypoxia in those areas may not be sufficient to cause necrosis. Results of a study40 in which human renal carcinoma cells were xenografted into nude mice indicate that bevacizumab treatment significantly retards tumor growth but has no effect on tumor necrosis. Alternatively, the observation period of the present study might have been insufficient for the tumors to outgrow their oxygen supply and become necrotic. Also, because of the large amount of variability in the percentage of tumor necrosis within tumors of the mice of the present study, the sample size might have been too small.
to detect a difference in percentage of tumor necrosis among the treatment groups.

Currently, there are no angiogenesis inhibitors approved by the FDA for the treatment of osteosarcoma. Results of studies of osteosarcoma in murine models indicate that IV administration of TNP-470 is a potent inhibitor of angiogenesis\textsuperscript{41} and IV administration of endostatin-coding lipid–DNA complexes delays tumor growth and prevents metastases to the lungs.\textsuperscript{42} However, to our knowledge, the present study was the first to demonstrate that IP administration of an angiogenesis inhibitor decreased microvessel density and slowed growth of canine osteosarcoma cells xenografted in mice.

Results of the present study suggested that administration of a VEGF inhibitor may be beneficial for the management of osteosarcoma. Secretion of VEGF by the HMPOS cells used for the xenografts of the present study was similar to that (1,440 pg/10\textsuperscript{6} cells) of phytohemagglutinin-stimulated peripheral blood mononuclear cells and markedly increased, compared with that (332 pg/10\textsuperscript{6} cells) of unstimulated peripheral blood mononuclear cells.\textsuperscript{43} For the present study, evaluation of direct binding and inhibition of canine VEGF by bevacizumab in vitro was not performed; therefore, it is possible that the decreased tumor growth associated with bevacizumab treatment was at least partially caused by inhibition of murine VEGF. Results of another study\textsuperscript{44} indicate that human and canine VEGF and VEGF receptors have a high degree of homology, which allows for functional exchange of VEGF. It also results in cross-reactivity such that a human VEGF assay could be used to quantify VEGF secretion by the canine HMPOS cells used in the present study. Because of the homology between human and canine VEGF, we believe that the treatment effects detected in the present study were the result of bevacizumab inhibition of canine VEGF.

Because bevacizumab is a monoclonal antibody of human origin, it could result in an anaphylactic reaction when administered to another species such as dogs. In the present study, no evidence of toxicity or anaphylaxis was detected in the mice treated with bevacizumab. In fact, at the time of euthanasia, the mean anaphylaxis was detected in the mice treated with bevacizumab. In the present study, no evidence of toxicity or anaphylaxis was detected in the mice treated with bevacizumab. However, it is possible that the decreased tumor growth associated with bevacizumab treatment was at least partially caused by inhibition of murine VEGF.

Traditionally, an antiangiogenic agent either suppresses the development of new vasculature within a tumor or destroys the existing tumor vasculature, although some agents suppress new development of as well as destroy existing tumor vasculature. The specific mechanism of action of an antiangiogenic agent should direct its use.\textsuperscript{31} For example, agents that suppress angiogenesis may be most beneficial for patients with micrometastatic disease or early-stage cancers,\textsuperscript{52–54} whereas agents that damage existing tumor vasculature may be most beneficial for patients with large solid tumors or late-stage cancers.\textsuperscript{55} Given the beneficial effects of antiangiogenic agents for the treatment of various neoplasias in dogs\textsuperscript{56} in addition to the results of the present study, evaluation of small-molecule angiogenic inhibitors for the treatment of osteosarcoma in dogs is warranted.

In the present study, we could not assess the effect of bevacizumab on metastatic disease because none of the mice developed metastatic disease as determined via necropsy and histologic evaluation. The absence of metastatic disease in the mice of the present study was likely caused by the fact that the HMPOS cells that were injected IM grew too rapidly and the mice were euthanized before metastasis had time to develop. Investigators\textsuperscript{45} who described the establishment of the HMPOS cell line used in the present study reported that micrometastasis in HMPOS-inoculated mice did not become evident until 4 weeks after inoculation and macrometastasis did not become evident until 6 weeks after inoculation. A murine model in which orthotopic tumor transplantation was followed by limb amputation would more closely mimic the clinical course of osteosarcoma and likely allow for evaluation of the effect of bevacizumab on metastatic disease.

Bevacizumab is a monoclonal antibody of human origin that has efficacy against various types of tumors in human patients, and a clinical trial is currently underway to evaluate its efficacy for the treatment of osteosarcoma in human patients.\textsuperscript{22–26,38} Results of the present study indicated that bevacizumab slowed the growth of canine osteosarcoma cells that were xenografted in mice in a dose-dependent manner. Given the homology of osteosarcoma.
between dogs and humans, the results of the present study might have important implications for the treatment of osteosarcoma in humans as well as in dogs. We concluded that the inhibition of VEGF as a treatment modality for osteosarcoma warrants further investigation.

References


