*Short Communication***Antitumor Effect of Bevacizumab in a Xenograft Model of Canine Hemangiopericytoma**

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Abstract. Canine hemangiopericytoma (CHP) is characterized by frequent local recurrence and increased invasiveness. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis in tumors. The aim of the present study was to investigate the effect of a single dose of bevacizumab on a xenograft model of CHP. VEGF protein was secreted from cultured CHP cells and interacted with bevacizumab. Bevacizumab treatment suppressed tumor growth by inhibiting tumor angiogenesis, whereas no significant differences were observed in the proliferation index and apoptosis rates of treated and untreated mice. Thus, bevacizumab had antitumor effects in a xenograft model of CHP.

Keywords: bevacizumab, canine hemangiopericytoma, vascular endothelial growth factor (VEGF)

Canine hemangiopericytoma (CHP) is a common mesenchymal tumor arising within the subcutaneous tissue. It frequently occurs in areas in the limbs where it is anatomically difficult to ensure a sufficient surgical margin (1). CHPs are characterized by frequent recurrence with increasing invasiveness following subsequent surgery. Therefore, aggressive initial surgery is considered the best treatment for CHP (1, 2). Radiation therapy is useful as an adjunct when complete removal is not possible, whereas chemotherapy is ineffective in CHP-bearing dogs (2, 3). Therefore, novel therapeutic approaches are necessary to improve the quality of life for dogs with advanced CHP.

Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis, growth, and metastasis in various tumors (4). Bevacizumab, a recombinant humanized monoclonal antibody against VEGF, has been clinically tested for the treatment of different cancers, including breast, lung, and colorectal cancer (5 – 7).

In veterinary medicine, VEGF is overexpressed in various cancers, including mammary tumors and CHP (8, 9). However, the antitumor effect of anti-angiogenic therapies remains unclear in tumor-bearing dogs. Thus, the aim of the present study was to evaluate the antitumor effect of bevacizumab in a xenograft model of CHP.

The cross-reactivity between bevacizumab (Avastin[®], Roche, Basel, Switzerland) and canine VEGF was confirmed by western blotting. Twenty micrograms of recombinant human VEGF (Peprotech, Rocky Hill, NJ, USA) and canine VEGF (R&D, Minneapolis, MN, USA) were analyzed by SDS-PAGE. The proteins were transferred onto PVDF membranes, which were blocked with 5% ECL blocking agent (GE Healthcare, Tokyo) in PBS and incubated overnight with bevacizumab at 4°C. The membranes were incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). The immunoreactivity was detected using an ECL plus western blotting detection kit (GE Healthcare) and LAS4000 luminescence imager (GE Healthcare).

A previously established CHP cell line was used in the present study (10). To determine whether CHP cells

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Fig. 1. Detection of vascular endothelial growth factor (VEGF) proteins in cultured CHP cells. A) Cross-reactivity with bevacizumab in canine VEGF. Lane 1, human VEGF; lane 2, canine VEGF. B) VEGF protein levels increased in a time-dependent manner in CHP cells cultured for 24 and 48 h. The differences were tested by Student's *t*-test and $P < 0.05$ was considered significant. C) Bevacizumab was localized in the pericytoplasm of CHP cells. Hematoxylin counterstaining. Bar = 50 μ m.

Fig. 2. Antitumor effect of bevacizumab in a xenograft model of CHP. A) Bevacizumab ($n = 7$, black square) or saline ($n = 6$, white square) was administered intraperitoneally twice per week for 21 days. The differences were tested by Student's *t*-test. $*P < 0.05$. Histological features of the tumors formed in NOG mice treated with bevacizumab (B) and saline (C). All tumors showed proliferation of the perivascular whorls of spindle tumor cells. H&E. Bar = 50 μ m. Immunohistochemical staining for FVIII in tumors treated with bevacizumab (D) and saline (E). Hematoxylin counterstain. Bar = 50 μ m.

secreted VEGF into the media, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Co., Ltd., Osaka) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) and 1% antibiotics (Invitrogen, Tokyo) for 24 and 48 h at 37°C in 5% CO₂. The media were

collected, centrifuged, and analyzed using a canine VEGF immunoassay kit (Quantikine, R&D), according to the manufacturer's instructions. The cross-reactivity between bevacizumab (Avastin®, Roche) and canine VEGF was confirmed by immunocytochemistry. The cultured cells were fixed with 4% paraformaldehyde

Fig. 3. Evaluation of microvessels, proliferation, and apoptosis in a CHP xenograft model. A) The density of FVIII-positive microvessels, B) indices of Ki-67-positive cells, and C) TUNEL-positive cells were evaluated by counting the positively-stained areas in a randomly selected high power field. The microvessel density was significantly lower in the bevacizumab-treated group compared with the control group. The differences were determined by Student's *t*-test. **P* < 0.05.

in PBS for 30 min at room temperature. After blocking with Block Ace (Dainippon Sumitomo Pharma, Osaka) containing 0.4% Triton X-100 for 30 min at room temperature, the cells were treated with bevacizumab (1:1000) and incubated with anti-human IgG (Zymed Laboratories Inc., South San Francisco, CA, USA) followed by the labeled streptavidin–biotin (LSAB) method. Finally, the cells were visualized with 3,3'-diaminobenzidine-4HCl (Dojindo, Kumamoto) and counterstained with hematoxylin.

To determine the antiproliferative effect of bevacizumab, 5×10^3 CHP cells were cultured in a 96-well flat-bottomed plate for 24 h and stimulated with culture medium containing four different doses (final concentration = 0.001, 0.01, 0.1, 1.0, or 10.0 $\mu\text{g/mL}$) of bevacizumab. The number of surviving cells was counted using Cell Counting Kit-8 (Dojindo) after 24, 48, and

72 h of treatment.

For the xenograft transplantation, a suspension of 5×10^5 viable CHP cells was injected subcutaneously into a 14-week-old, non-obese diabetic (NOD) / Shi-scid IL2R γ^{null} (NOG) male mouse, obtained from the Central Institute for Experimental Animals (Kawasaki). After tumor formation was confirmed macroscopically, saline ($n = 6/\text{group}$) or bevacizumab (4.0 mg/kg; $n = 6/\text{group}$) was administered intraperitoneally twice per week for 21 days. The dose of bevacizumab was determined based on a previous report (11). The tumor volume (*V*) was estimated using the equation, $V = (\text{length}) \times (\text{width})^2 / 2$. Tumors formed in NOG mice were fixed with 10% neutral buffered formalin and routinely embedded in paraffin wax. The cut sections were stained with hematoxylin and eosin (H&E). Serial sections were immunostained by the LSAB method using mouse monoclonal antibodies against factor VIII-related antigen (FVIII, clone F8/86; Nichirei Biosciences, Tokyo) and Ki-67 (clone MIB-1; DAKO A/S, Glostrup, Denmark). The sections were also treated with a commercial ApopTag peroxidase in situ Apoptosis Detection kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The densities of the microvessels and the proliferation and apoptosis indices were evaluated with FVIII using the hot-spot method (12). Briefly, slides were examined at low power ($\times 40$) to identify 10 areas with high vessel densities and these areas were further evaluated at high power ($\times 400$). The mean number of vessels per 10 fields was determined. Continuous vessels were counted as one vessel. A similar method was used to evaluate Ki-67- or TUNEL-positive cells for proliferation or apoptosis, respectively. All the experiments were approved by the Animal Experiments Committee at the Nippon Veterinary and Life Science University.

Bevacizumab was detected at the expected molecular weight of 19 kDa corresponding to human and canine VEGFs (Fig. 1A). VEGF protein levels (mean $\pm$ standard deviation) in the media were 47.160 ± 5.630 and 88.632 ± 5.458 ng/mL after cells were cultured for 24 and 48 h, respectively (Fig. 1B), whereas VEGF protein was not detected in the medium without the cells. We also confirmed the interaction between canine VEGF secreted from cultured CHP cells and bevacizumab. Immunocytochemically, bevacizumab was localized in the pericytoplasm of cultured CHP cells (Fig. 1C). These results indicated that VEGF was secreted from CHP cells and interacted with bevacizumab. The proliferation of CHP cells was not significantly different in bevacizumab-treated and untreated conditions (data not shown).

The antitumor activity of bevacizumab was evaluated using a xenograft model of CHP. Aggressive tumor growth was significantly suppressed in the bevacizumab-

treated group compared with the control group (Fig. 2A). No abnormal clinical signs were observed during the follow-up. Histologically, all the induced tumors showed proliferation of the perivascular whorls of spindle tumor cells (Fig. 2: B and C), which was consistent with a previous study (10). No metastases of the tumor cells were observed. The density of FVIII-positive microvessels in the formed tumors was significantly lower in the bevacizumab-treated group than in the control group (Fig. 2: D, E, and Fig. 3A), whereas no significant differences were observed in the proliferation and apoptosis indices (Fig. 3: B and C).

VEGF has been associated with multiple aspects of tumor growth including angiogenesis, proliferation, and apoptosis inhibition. Bevacizumab is known to interact specifically with human VEGF but not murine VEGF (13). Furthermore, the one binding site for VEGF is conserved in humans and dogs (GenBank Accession No. NP_001003175). In the present study, we demonstrated that bevacizumab interacted with canine VEGF and inhibited tumor growth by suppressing tumor angiogenesis in a xenograft model of CHP. However, bevacizumab did not show antiproliferative activity, and it did not enhance apoptosis in a xenograft mouse model. Similar effects of bevacizumab treatment as a single agent have been reported in xenograft models of human glioma U251 and colon cancer KM12SM cell lines, whereas its combined administration with irinotecan enhanced apoptosis and reduced the proliferation index (14, 15). The antitumor effects of bevacizumab in combination with chemotherapeutic drugs were not evaluated in the present study.

In human medicine, the clinical efficacy of bevacizumab in combination with certain chemotherapies has been reported for the treatment of various cancers, such as colon and breast cancer (5, 6). Whether a similar antiproliferative effect occurs in CHP-bearing dogs remains unclear, although bevacizumab showed promising results in CHP xenograft mice. The potential for adverse clinical effects including anaphylactic shock, production of neutralizing antibodies, or rejection in CHP-bearing dogs treated with bevacizumab should be considered. Further studies are needed to clarify the effects of bevacizumab in CHP-bearing dogs.

In conclusion, bevacizumab demonstrated antitumor effects in a xenograft model of CHP. This study is the first to assess the antitumor effect of bevacizumab in a xenograft model of CHP and has possible applications in veterinary medicine. Bevacizumab may be useful for the treatment of CHP and other tumors with VEGF overexpression.

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