Anti-tumor effect of bevacizumab on a xenograft model of feline mammary carcinoma

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(Received 17 September 2015/Accepted 17 November 2015/Published online in J-STAGE 28 November 2015)

ABSTRACT. Feline mammary carcinomas are characterized by rapid progression and metastases. Vascular endothelial growth factor (VEGF) is a key regulator of tumor angiogenesis, proliferation and metastasis. The present study aimed to investigate the effects of a single drug therapy of bevacizumab on a xenograft model of feline mammary carcinoma expressing VEGF protein. Bevacizumab treatment suppressed tumor growth by inhibiting angiogenesis and enhancing apoptosis; however, it did not affect the tumor proliferation index. Thus, bevacizumab had anti-tumor effects on a xenograft model, and this may be useful for the treatment of feline mammary carcinoma.

KEYWORDS: bevacizumab, feline mammary carcinoma, vascular endothelial growth factor (VEGF), xenograft

doi: 10.1292/jvms.15-0550; J. Vet. Med. Sci. 78(4): 685–689, 2016

Mammary carcinoma is the third most common cancer in cats, mainly affecting females with a mean age of 10–12 years at diagnosis. More than 80% of feline mammary tumors show considerable malignancy along with rapid progression and metastasis to the lung and local lymph nodes at an early stage [23]. Therefore, aggressive initial surgery is considered the best treatment for feline mammary carcinoma. Adjuvant chemotherapy using cyclophosphamide, vincristine and doxorubicin is not useful for the treatment of feline mammary carcinomas [10, 14]. Therefore, novel therapeutic strategies are required to improve the quality of life in these cats. Specific therapies targeting molecules, such as cyclooxygenase-2, are being developed for treating feline mammary carcinomas [3]. However, at present, there is no established effective treatment for feline mammary carcinomas.

Tumor angiogenesis plays a critical role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis, growth and metastasis in various tumors [2]. VEGF receptors, such as VEGFR-1/fms-like tyrosine kinase-1 (Flt-1) and VEGFR-2/kinase insert domain receptor (KDR), bind to VEGF with high affinity, but only VEGFR-2 is capable of mediating angiogenesis [4, 21]. VEGFR-1 decreases the availability of VEGF to VEGFR-2 and plays a negative regulatory role in angiogenesis [5]. Bevacizumab, a recombinant humanized monoclonal antibody against VEGF, has been clinically tested for the treatment of different cancers in humans. Bevacizumab has been shown to specifically interact with human VEGF but not with mouse and rat VEGF [19]. Some veterinary studies have reported that bevacizumab and VEGF receptor tyrosine kinase (RTK) inhibitors, such as sunitinib, may be useful for the treatment of companion animals with tumors [15, 20]. On the other hand, the therapeutic potential application of the new recombinant oncocytic vaccinia virus GLV-5b451 expressing the anti-VEGF single-chain antibody GLAF-2 has also been evaluated in a xenograft model for feline mammary carcinoma [1]. In cats, previous studies have demonstrated that VEGF is expressed in various cancers, including mammary carcinomas, and its expression is significantly correlated with tumor grading and a short survival period. Furthermore, the co-expression of VEGF and KDR may reflect the existence of possible autocrine and paracrine loops between epithelial, endothelial and/or stromal cells in feline mammary carcinoma [17]. However, the anti-tumor effects of these anti-angiogenic therapies in preclinical studies remain unclear.

One binding motif for VEGF receptor was conserved between human and feline VEGFs [12]. In this study, we investigated the anti-tumor effect of bevacizumab on a xenograft model of feline mammary carcinoma.

A feline mammary carcinoma cell line FKNp previously was used in the present study [22]. Cells were maintained in RPMI 1640 (Wako Pure Chemical Co., Ltd., Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, UT, U.S.A.) and 1× antibiotic/antimycotic solution (Nakarai Tesque, Kyoto, Japan), and incubated at 37°C in an atmosphere containing 5% CO₂.
Cells were cultured, washed with PBS and lysed in RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% Triton X-100] containing a protease inhibitor cocktail (Complete; Roche Diagnostics, Tokyo, Japan). Twenty μg of sonicated lysate from FKNp or recombinant feline VEGF (R&D, Minneapolis, MN, U.S.A.) were analyzed by SDS-PAGE. The proteins were transferred onto PVDF membranes, which were blocked using 5% ECL blocking agent, (GE Healthcare, Tokyo, Japan) in PBS and incubated overnight at 4°C with bevacizumab and the following antibodies: VEGF (Santa Cruz Biochemistry, Santa Cruz, CA, U.S.A.), VEGFR-1 (Flk-1, Santa Cruz Biochemistry) and VEGFR-2 (Flk-1/KDR, Santa Cruz Biochemistry). The membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, U.S.A.) for bevacizumab and HRP-conjugated anti-rabbit IgG (Santa Cruz Biochemistry) for others. The immunoreactivity was detected using an ECL plus Western blotting detection kit (GE Healthcare) and LAS4000 luminescence imager (GE Healthcare).

To determine the antiproliferative effect of bevacizumab, 5 × 10^3 FKNp cells were cultured in a 96-well flat-bottomed plate for 24 hr and stimulated with culture medium containing five different doses (final concentration=0.001, 0.01, 0.1, 1.0 or 10.0 μg/ml) of bevacizumab. The number of surviving cells was counted using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) after 24, 48 and 72 hr of treatment. Absorbance was measured for each well at a wavelength of 450 nm. To examine the cytotoxic effect of bevacizumab, 5 × 10^3 FKNp cells were cultured in a 96-well flat-bottomed plate (PerkinElmer, Waltham, MA, U.S.A.) and stimulated with bevacizumab in condition as above described. After 48 hr of incubation, FKNp cells were analyzed with CytoTox-Glo cytotoxicity assay (Promega, Madison, WI, U.S.A.), according to the manufacturer’s instructions. Dead-cell luminescence was measured by GloMax multi detection system (Promega).

For the xenograft transplantation, a suspension of 5 × 10^5 viable FKNp cells was injected subcutaneously into 14-week-old, nonobese diabetic (NOD)/Shi-scid IL2Rγnull (NOG) male mice, obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). After tumor formation was macroscopically confirmed, we administered saline (n=4/group) or bevacizumab (4.0 mg/kg; n=4/group) intraperitoneally twice a week for 28 days. The volume of bevacizumab was determined on the basis of a previous report [15]. The tumor volume (V) was estimated using the equation V=(length) ×(width)^2/2. All experiments were approved by the Animal Experiments Committee of the Nippon Veterinary and Life Science University.

Tumors that formed in NOG mice were fixed with 10% neutral-buffered formalin and routinely embedded in paraffin wax. The cut sections (4 μm) were stained with hematoxylin and eosin (H&E). Serial sections were immunostained by the LSAB method using mouse monoclonal antibodies against Ki67 (Dako, Glostrup, Denmark) and α smooth muscle actin (αSMA, Dako), and rabbit polyclonal antibody against VEGF (Santa Cruz Biochemistry). The sections were also treated with a commercial ApoTag peroxidase in situ apoptosis detection kit (Millipore, Billerica, MA, U.S.A.), according to the manufacturer’s instructions. The density of the microvessels, proliferation and apoptotic indices were evaluated for αSMA by the hot-spot method [15]. In brief, slides were examined under low power (40×) to identify 10 areas with high-vessel densities, and these areas were further evaluated under high power (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 Ki67- or TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells for proliferation or apoptosis, respectively. Significance differences were determined using the Student’s t-test. P values of <0.05 were considered to be significant.

Western blotting showed that FKNp expressed VEGF, VEGFR-1 and VEGFR-2 (Fig.1A), suggesting the possible association between VEGF and its receptors in FKNp. Bevacizumab was detected at the expected molecular weight of 21 and 42 kDa, corresponding to VEGF monomer and dimer, respectively, in extracts prepared from FKNp cells as well as with recombinant feline VEGF monomer (Fig. 1B). These results indicate that bevacizumab interacts with feline VEGF secreted from FKNp. The proliferation and cytotoxicity of FKNp cells were not significantly different in bevacizumab-treated and untreated conditions (data not shown).

The anti-tumor activity of bevacizumab was evaluated using a xenograft model of feline mammary carcinoma. Compared with the control group, aggressive tumor growth was significantly suppressed in the bevacizumab-treated group (Fig. 2). No abnormal clinical signs were observed during the follow-up period. On histological examination of the mice, the induced tumors showed proliferation with tubulopapillary patterns and a solid pattern with central necrosis (Fig. 3A and 3B). Histological features were similar in bevacizumab-treated and control groups. The tumor cells were strongly positive for VEGF (Fig. 3C and 3D). We did not observe metastasis of tumor cells to other organs, including the neighboring lymph nodes, lung or liver. The density of αSMA-positive microvessels in the formed tumors was significantly lower in the bevacizumab-treated group than in the control group (Fig. 4A–4C). The apoptotic index was significantly higher in the bevacizumab-treated group than in the control group (Fig. 4D–4F). No significant difference was observed in the proliferation index between the two groups (Fig. 4G–4I).

VEGF plays an important role in tumor growth, including angiogenesis, proliferation and inhibition of apoptosis mediated by autocrine or paracrine loops between VEGF and its receptors. In the present study, bevacizumab treatment inhibited tumor growth by suppressing tumor angiogenesis and enhancing apoptosis in a xenograft model of feline mammary carcinoma expressing VEGF. However, bevacizumab did not show anti-proliferative activity. Similar to our results, Fujita et al. reported the anti-tumor effects mediated by decreasing microvessels and increasing apoptosis in xenograft models of human squamous cell carcinoma [6]. Similar effects of a single drug therapy of bevacizumab have been reported in xenografts of human cancers, includ-
Bevacizumab interacts with VEGF from FKNp line. (A) Cell extracts from FKNp and recombinant feline VEGF (rfVEGF) were analyzed by immunoblotting with anti-VEGFR-1 and anti-VEGFR-2 (upper) and anti-VEGF (bottom) antibodies. Anti-VEGF antibody detected VEGF monomer 21 kDa and dimer 42 kDa in the FKNp as well as rfVEGF monomer. (B) Bevacizumab interacts with VEGF monomer 21 kDa and dimer 42 kDa, and rfVEGF monomer.

VEGF proteins are dimeric molecules that exist in multi-forms due to alternative splicing [7]. In feline mammary tumors, the overexpression of VEGF was found to be related to tumor grading and angiogenesis by using immunohistochemistry [16]. In the present study, bevacizumab was strongly interacted to VEGF dimer than monomer in FKNp line, suggesting that FKNp line contains a high proportion of dimeric VEGF.

Several anti-tumor drugs, such as doxorubicin, mitoxantrone, vincristine and cisplatin, have been evaluated for their anti-proliferative effects on feline mammary carcinoma cell lines. The cell lines were sensitive to the inhibitory effects of these drugs, although the responses varied [18]. In feline mammary carcinomas, adjuvant chemotherapy using vincristine and doxorubicin has not been established [10, 14]. However, the anti-tumor effects of chemotherapeutic drugs may be elicited by intratumor anti-angiogenesis and vascular normalization through decreasing VEGF [11].

Small-molecule tyrosine kinase inhibitors that inhibit target-specific receptor TKs (RTKs) and monoclonal antibodies that inhibit a wide spectrum of RTKs have been extensively used in human medicine. Anti-angiogenic therapies mediated by bevacizumab and VEGF RTK inhibitors, including sunitinib, result in tumor regression and may enhance the effects of chemotherapy [8]. In veterinary medicine, two small molecules, toceranib (Palladia®) and masitinib (Masivet®), have recently been approved by the European Medicines Agency and the American Food and Drug Administration for use in dogs with mast cell tumors [13, 20]. Toceranib, a multikinase inhibitor, which targets Kit, platelet-derived growth factor receptor and VEGFR-2, is approved for use in recurrent, nonresectable grade II/III mast cell tumors. It also appears to possess biological activity against other tumors,
Fig. 3. The anti-tumor effects of bevacizumab in a xenograft model of feline mammary carcinoma. The histological features of the tumors formed in NOG mice treated with saline (A) and bevacizumab (B). The induced tumors showed proliferation with tubulopapillary patterns and a solid pattern. H&E. The immunohistochemical staining for VEGF in tumors treated with saline (C) and bevacizumab (D). Hematoxylin counterstain. Bar=50 µm.

Fig. 4. Evaluation of microvessel formation, apoptosis and proliferation in an FKNp xenograft model. Immunohistological features of the tumors formed in NOG mice treated with saline (A, D and G) and bevacizumab (B, E and H). The density of αSMA-positive microvessels (A–C), indices of TUNEL-positive cells (D–F) and Ki67-positive cells (G–I) were evaluated by counting the positively stained areas in a randomly selected high-power field. The microvessel density and apoptotic index were significantly different between the bevacizumab-treated and control groups. The differences were tested using the Student’s t-test. *P<0.05. Hematoxylin (A, B, G and H) and methylgreen (D and E) counterstain. Bar=50 µm.
such as soft tissue sarcoma and mammary carcinoma, in dogs [20]. In preclinical studies, a single administration of bevacizumab demonstrated anti-tumor effects on a xenograft model of canine hemangiopericytoma [15]. However, the effects of molecular target therapy against angiogenesis in feline tumors remain elusive. Further studies are required to develop anti-angiogenic therapies mediated by TK inhibitors as well as bevacizumab.

In summary, bevacizumab treatment suppressed tumor growth in a xenograft model of feline mammary carcinoma expressing VEGF. Therefore, bevacizumab may be useful for the treatment of feline mammary carcinoma with careful consideration of the potential adverse clinical effects.

ACKNOWLEDGMENT. This work was supported in part by the Strategic Research Base Development Program for Private Universities from Ministry of Education, Cultures, Sports, Science and Technology of Japan (MEXT), 2008–2012, and a Grant-in-aid for Scientific Research (No. 24248050, No. 24780314 and 26292161 to M. Michishita) from the MEXT.

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