Research in Veterinary Science 122 (2019) 1–6

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Vascular disrupting effect of combretastatin A-4 phosphate with inhibition of vascular endothelial cadherin in canine osteosarcoma-xenografted mice

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ARTICLE INFO

Keywords:
Vascular disrupting agent
Combretastatin A-4 phosphate
Tumor angiogenesis
VE-cadherin
Osteosarcoma

ABSTRACT

Combretastatin A-4 phosphate (CA4P) induces tumor necrosis by selectively inhibiting tumor blood flow. However, the detailed mechanisms by which CA4P selectively disrupts tumor blood vessels are not well understood. Our previous study indicated that the selective blocking effect of CA4P might be related to a vascular endothelial cadherin (VE-cadherin) dysfunction in the tumor vasculature. In this study, we evaluated the vascular disrupting effect of CA4P on canine osteosarcomas xenografted into mice, focusing on VE-cadherin. Even though 30 mg/kg CA4P only partially inhibited blood flow in the xenografted tumor, a combination of an anti-VE-cadherin neutralizing antibody and 30 mg/kg CA4P inhibited most of the tumor blood flow. In addition, the combination of antibody and drug significantly inhibited tumor growth compared to the control. These results strongly suggested a relationship between the expression of VE-cadherin in tumor blood vessels and the selective blocking mechanisms of CA4P.

1. Introduction

Tumor vasculature is necessary for tumor growth because it supplies nutrients and oxygen to the tissues. Antivascular drugs inhibit blood supply to tumor tissues, leading to tumor necrosis. Several antivascular agents have been developed in both human and veterinary medicine, including bevacizumab, sunitinib, and toceranib (Lin et al., 2016; Ranieri et al., 2013). These drugs are called antiangiogenic agents and inhibit neovascularization processes in tumor tissues. Combretastatin A-4 phosphate (CA4P) is classified as a vascular disrupting agent (VDA) that selectively blocks established tumor vasculature within a few hours (Liang et al., 2016). However, the detailed mechanisms by which CA4P specifically affects tumor blood vessels are not well understood (Vincent et al., 2005).

Tumor blood vessels have various specific features that differ from those of normal blood vessels. They are morphologically different, being more tortuous and leaky (Ruoslahti, 2002), and their endothelial junctions are more vulnerable than those of normal blood vessels (Dudley, 2012). Moreover, tumor tissue-derived endothelial cells overexpress specific markers such as VEGFR2 and CXCR7, have cytogenetic abnormalities, and are resistant to different anticancer drugs (Hida et al., 2004; Ohga et al., 2012). These abnormal characteristics of tumor vasculature may be responsible for the selective tumor vascular blocking mechanisms of CA4P.

In our previous study, canine tumor tissue-derived endothelial cells (TECs) were found to be more susceptible to CA4P than canine normal tissue-derived endothelial cells (NECs) and it was suggested that the vascular endothelial cadherin (VE-cadherin) in TECs might have functional abnormalities (Izumi et al., 2017). In addition, inhibition of VE-cadherin in NECs increased the susceptibility of the cells to CA4P (Izumi et al., 2017). These results indicate that the fragility of VE-cadherin in TECs may be the cause of the selective blocking mechanisms of CA4P.

Even though several VDAs are currently in clinical trials in human medicine (Liang et al., 2016), there are few reports on CA4P in veterinary fields (Abma et al., 2017a, 2017b). By investigating the blood flow blocking effect of CA4P using canine tumor-xenografted mice, it will be possible to obtain fundamental knowledge for future clinical applications of CA4P in veterinary fields. Therefore, in this study, we examined the vascular disrupting effect of CA4P in canine tumor-xenografted mice, focusing on VE-cadherin.

2. Materials and methods

2.1. Tumor model in nude mice

Animal experiments were approved by the Institutional Animal Care and Use Committee of the National University Corporation, Hokkaido

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https://doi.org/10.1016/j.rvsc.2018.10.017
Received 1 March 2018; Received in revised form 29 October 2018; Accepted 29 October 2018

University (permit number 16–0096). The canine osteosarcoma cell line HMPOS (Barroga et al., 1999) was cultured at 37 °C in a humidified atmosphere, with 5% CO_2. Dulbecco's Modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, was used. Tumor cells (5×10^5 in 500 μL phosphate-buffered saline, PBS) were implanted subcutaneously into the rear dorsum of 4-week-old male nude mice (BALB/cAJcl-nu/nu; CLEA Japan, Inc., Tokyo, Japan). The mice were housed under enriched environmental conditions with free access to food and water.

2.2. Drug treatment

CA4P was purchased from Selleck Chemicals LLC (Houston, TX, USA), dissolved in PBS, and stored at −80 °C until use. To examine the blood flow blocking effect of CA4P, mice were divided into five groups (n = 6 for each group) and injected intraperitoneally with the following solutions: (1) PBS (control group); (2) 30 mg/kg CA4P; (3) VE-cadherin neutralizing antibody (VEC NAb; 40 μg/mouse, Thermo Fisher Scientific, Inc., Waltham, MA, USA); (4) 30 mg/kg CA4P and VEC NAb (40 μg/mouse) (combination group); and (5) 100 mg/kg CA4P. Mice were sacrificed using carbon dioxide gas 3 h after CA4P treatment and 24 h after VEC NAb treatment. In the combination group, CA4P treatment was administered 21 h after VEC NAb. The drugs were administered when the tumor diameter reached about 10 mm. In order to evaluate vascular perfusion, tumor tissues and normal tissues (brain, lung, heart, liver, and kidney) were resected and used for immunofluorescence microscopy analysis.

To examine CA4P's antitumor effect, mice were divided into four groups (n = 5 for each group) and injected intraperitoneally with the following solutions: (1) PBS (control group); (2) 30 mg/kg CA4P; (3) VEC NAb (40 μg/mouse); and (4) 30 mg/kg CA4P and VEC NAb (40 μg/mouse) (combination group). VEC NAb was administered on days 12 and 15 after tumor transplantation and CA4P was administered 21 h after VEC NAb (days 13 and 16). Tumor size was measured every other day. Tumor volume was calculated using a modified ellipsoidal formula (Euhus et al., 1986; Jensen et al., 2008): Tumor volume = 1/2 (length × width^2).

Since there was a high variation in tumor volume on day 12 after implantation, the size of the tumor in each mouse was expressed as a relative value:

$$\text{Relative tumor volume} = \text{daily measured tumor volume} / \text{day 12 tumor volume}$$

2.3. Immunofluorescence microscopy analysis and vascular perfusion

The blood flow blocking effect of CA4P was investigated using the methods described in the previous study, with some modifications (Williams et al., 2014). Briefly, each mouse was injected intravenously with 100 μg Dylight 488-conjugated tomato lectin in 200 μL PBS. Mice were sacrificed 10 min later, and tumor and normal tissues were excised (brain, heart, lung, liver, and kidney), embedded in optimal cutting temperature compound, and rapidly frozen using liquid nitrogen. Cryostat sections of tumor (30 μm thick) and normal tissues (10 μm thick) were fixed in 4% paraformaldehyde, and then blocked using 5% goat serum in PBS. We examined the condition beforehand and selected the thickness of the section where the blood vessel was easiest to observe. These samples were incubated with rat anti-CD31 antibody (1:500 dilution, BD Pharmingen, Oxford, UK) for 1 h at room temperature. After washing, sections were further incubated with Alexa-Fluor 555-conjugated goat anti-rat antibody (1:500 dilution, Invitrogen Molecular Probes, Paisley, UK) for 1 h at room temperature. The sections were then washed three times with PBS and mounted with Prolong Gold Antifade mountant with 4′,6-diamidino-2-phenylindole (Invitrogen Life Technology, Gaithersburg, MD, USA). Immunofluorescence images were obtained by confocal microscopy (LSM 700; Zeiss, Oberkochen, Germany).

Tomato lectin-positive and CD31-positive areas were counted (number of pixels) using Image J software (http://rsb.info.nih.gov/ij/, National Institutes of Health, Bethesda, Maryland, USA). Since any blockages in blood flow would not allow the inflow of tomato lectin after intravenous administration, vascular perfusion could be calculated by the following equation:

$$\text{Vascular perfusion} = \text{tomato lectin} - \text{positive area/CD31} - \text{positive area}$$

2.4. Statistical analysis

Multiple comparisons were performed using Tukey's tests. The results are expressed as means, with error bars indicating standard deviation. Values of $P < .05$ were considered significant. All statistical analyses were performed using EZR software (Kanda, 2013).

3. Results

3.1. Vascular disrupting effects of CA4P

In the control group, no blood flow blocking (tomato lectin negative and CD31 positive) was observed in the tumor tissue (Fig. 1). In the group that was administered 30 mg/kg CA4P, partial blood flow blocking in the tumor tissue was observed in four of the six mice (Fig. 1), but not in the other two (Fig.S1). In the group treated with 40 μg VEC NAb, no blood flow blocking was observed in the tumor tissues (Fig. 1). In the combination group (30 mg/kg CA4P and 40 μg VEC Nab), blood flow in most of the tumor tissue was blocked (Fig. 1); in addition, no vascular disruption was observed in adipose tissue around the tumor tissue (Fig. S2). In the group that was administered 100 mg/kg CA4P, blood flow in most tumor tissue was also blocked (Fig. 1). No blood blocking effects were detected in the normal tissues of mice in any of the groups (Fig. 2).

There was a tendency towards lower vascular perfusion (0.83 ± 0.17; mean ± SD) in the group that was administered 30 mg/kg CA4P than in the control group (1.03 ± 0.15), but no significant difference was observed (Fig. 3). Vascular perfusion in the group that was administered 40 μg VEC NAb (0.99 ± 0.19) was almost equal to that of the control group (Fig. 3). The combination group (0.28 ± 0.26) and 100 mg/kg CA4P group (0.31 ± 0.17) showed similar decreases in vascular perfusion. Vascular perfusion of both groups was significantly lower than the other three groups.

3.2. Antitumor effect of CA4P

The relative tumor volume was significantly lower in the combination group than in the control group (Fig. 4). The rate of increase of tumor volume tended to be lower in the combination group than in those groups treated with VEC NAb or 30 mg/kg CA4P; however, no significant differences were observed (Fig. 4). Relative tumor volume was significantly lower in the group administered 30 mg/kg CA4P than in the control group (Fig. 4). There were no significant differences between the other groups.

4. Discussion

In this study, we focused on VE-cadherin to evaluate the tumor vascular disrupting effects of CA4P in canine osteosarcoma-xenografted mice. VE-cadherin is a vascular endothelial-specific intercellular adhesion molecule that is found at intercellular junctions and has the function of maintaining intercellular interactions and cell morphology (Liao et al., 2002). In this study, anti-VE-cadherin monoclonal antibody BV13 was used to neutralize VE-cadherin. BV13 disrupts VE-cadherin

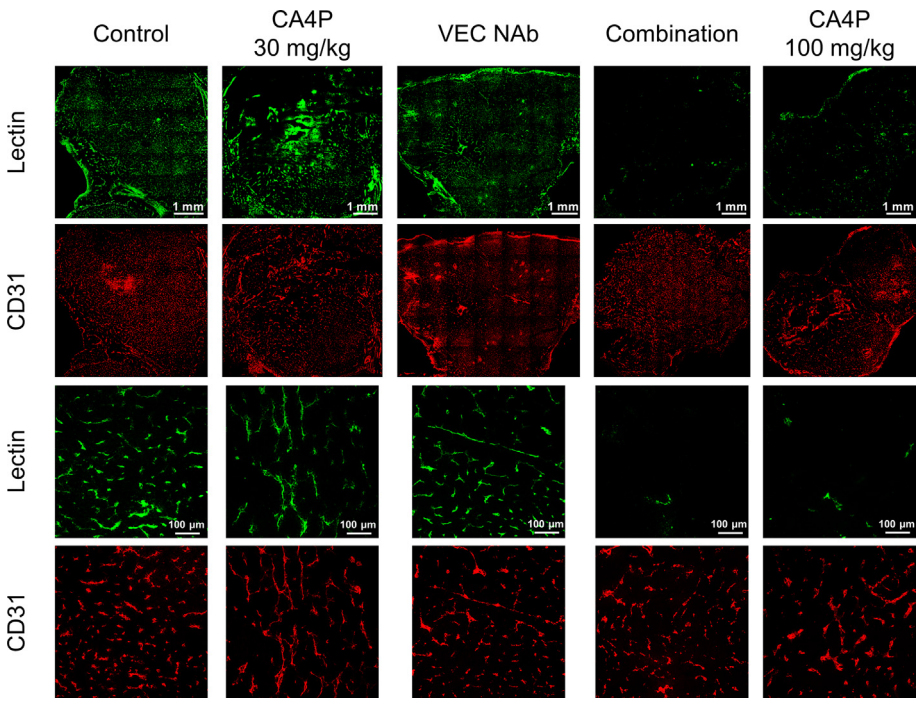

Fig. 1. Immunofluorescence analysis of vascular disrupting effects in tumor tissues.
Representative images of Dylight 488-tomato lectin (pseudo-colored green) and Alexa-fluor 555-CD31 (pseudo-colored red) immuno-stained frozen sections from xenografted canine osteosarcoma tumors in the different treatment groups: control; combretastatin A-4 phosphate (CA4P, 30 mg/kg); VE-cadherin neutralizing antibody (VEC NAb, 40 μg/mouse); combination treatment (30 mg/kg CA4P 21 h after 40 μg VEC NAb); and CA4P (100 mg/kg). The upper half of this figure shows the image of the whole tumor and the lower half shows a magnified image. Tumors were excised 3 h after CA4P treatment or 24 h after VEC NAb treatment. Scale bars represent 1 mm or 100 μm.

homotypic adhesion and clustering in rodents (Corada et al., 1999) and increases vascular permeability and inhibits angiogenesis, which produces an antitumor effect (Corada et al., 2002; Liang et al., 2016). In our previous study on canine endothelial cells, VE-cadherin in tumor vessels was suggested to have functional abnormalities, making it vulnerable to the contractile effects induced by CA4P. This fragility in VE-cadherin might be responsible for the selective blocking mechanisms of CA4P (Izumi et al., 2017). It was expected that further blood flow blocking effects would be obtained if CA4P was administered after inhibiting tumor VE-cadherin *in vivo*.

Our analyses in this study showed that 30 mg/kg CA4P only partially blocked the blood flow within the tumor; however, in combination with VEC NAb, most blood flow in the tumor tissues was blocked to the same extent as that after 100 mg/kg CA4P. In previous studies, CA4P was administered to tumor-xenografted mice at a concentration of 100 mg/kg, which disrupted most of the blood flow in the tumor tissue (Tozer et al., 1999; Williams et al., 2014). In this study, 100 mg/kg CA4P also induced blood flow blocking effects. We also found that the effect of CA4P was enhanced by inhibiting VE-cadherin *in vivo*; this observation is consistent with the results from previous *in vitro* experiments (Izumi et al., 2017). Considering the past report (Izumi et al., 2017), these results indicated that fragile VE-cadherin in tumor blood vessels became more vulnerable after the administration of neutralizing VE-cadherin antibodies and became more susceptible to

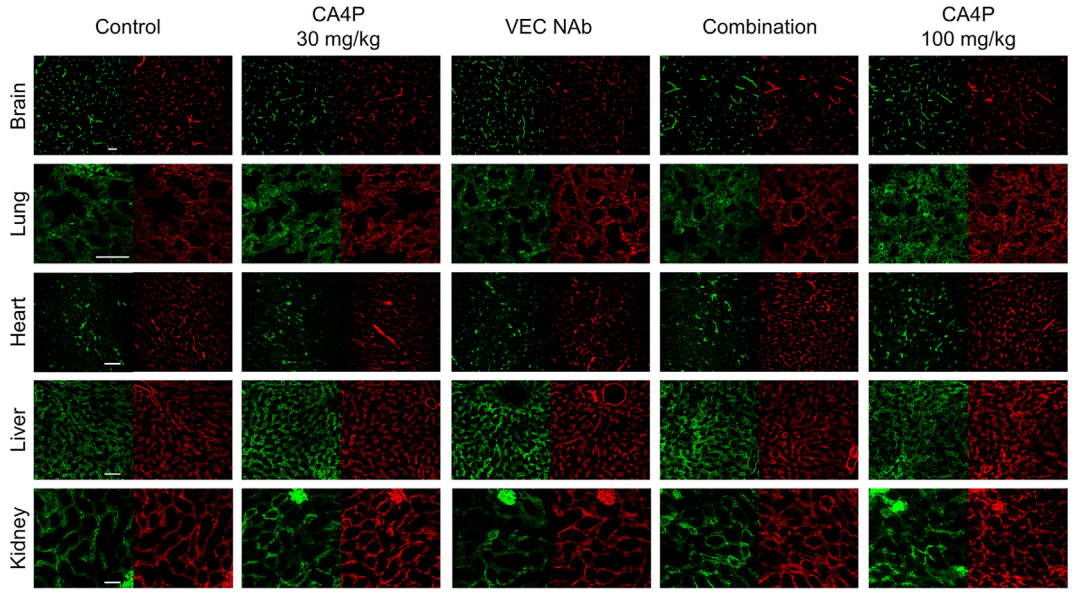

Fig. 2. Immunofluorescence analysis of vascular disrupting effects on normal tissues.
Representative images of Dylight 488-tomato lectin (pseudo-color green) and Alexa-fluor 555-CD31 (pseudo-color red) immuno-stained frozen sections from normal tissues of mice in the different treatment groups: control; CA4P (30 mg/kg); VE-cadherin neutralizing antibody (40 μg/mouse); combination treatment (30 mg/kg CA4P 21 h after 40 μg VEC NAb); and CA4P (100 mg/kg); Tissues were excised 3 h after CA4P treatment or 24 h after VEC NAb treatment. Scale bars represent 50 μm.

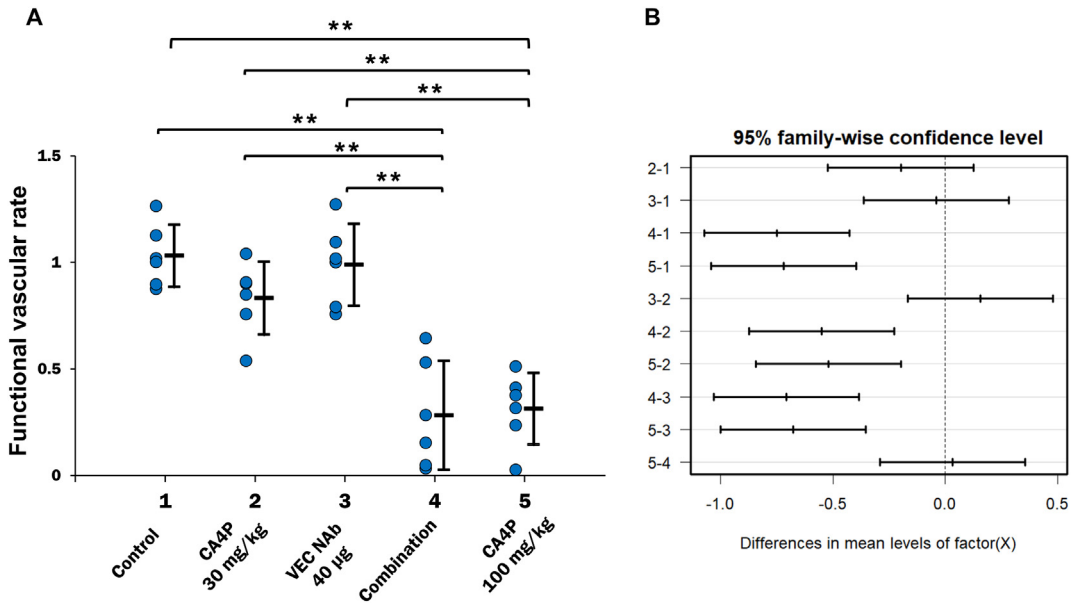

Fig. 3. Analysis of the vascular perfusion.
(A) Vascular perfusion was calculated using the equation: number of pixels positive for tomato lectin/number of pixels positive for CD31. Dot plots in each group represent the vascular perfusion of tumor tissue for each mouse. Error bars represents means ± SD for n = 6. P < .05 was considered significant, Tukey's test, **P < .01. (B) 95% family-wise confidence level (the 95% confidence interval of the differences in the mean value of vascular perfusion between groups in this study). The different treatment groups are: 1, control; 2, CA4P (30 mg/kg); 3, VEC NAb (40 μg); 4, combination group (30 mg/kg CA4P and 40 μg VEC NAb); and 5, CA4P 100 mg/kg.

the effects of CA4P; as a consequence, the blood flow blocking effect was enhanced.

Normal tissues did not contain any regions with blood flow blocking in any of the treatment groups. Blood flow in normal tissues might be blocked by a combination treatment in which the concentrations of CA4P and/or VEC NAb are increased. One limitation of the present study is that the blood flow blocking effect was only examined at 3 h after CA4P administration. Although no obvious side effects were observed in the mice, partial blood flow blockage might occur if the observation time after administration was changed. The influence of changing the administration dose of the drugs or the observation time of normal tissues needs to be investigated in future experiments.

According to the previous study (Barroga et al., 1999), HMPOS cells growth very rapidly and metastasized to the lung of xenografted mouse. Macroscopic metastasis of HMPOS cells was observed 6 weeks after tumor transplantation, but not confirmed in 4 weeks. Small amounts of

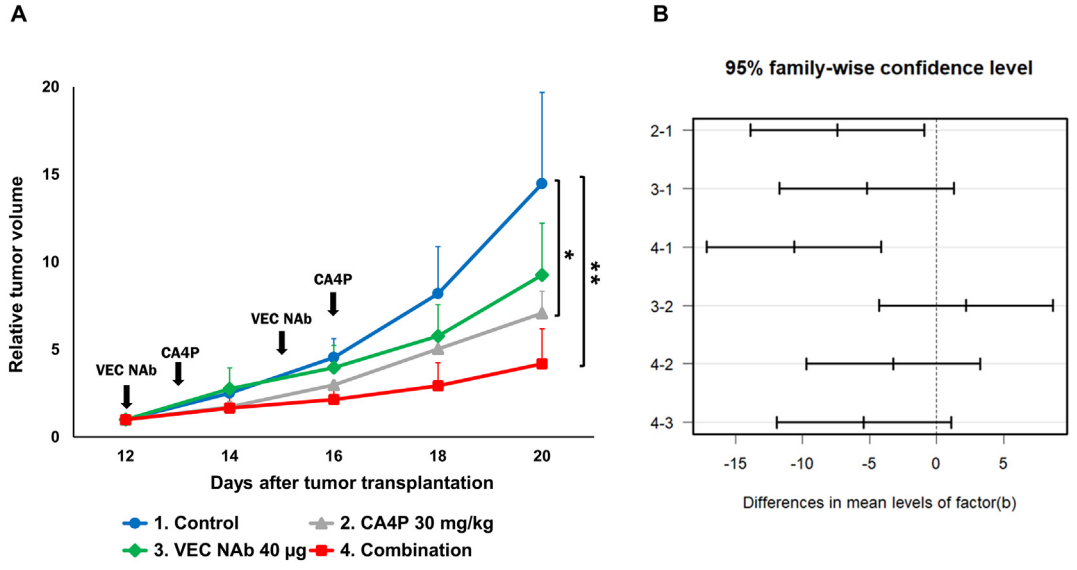

Fig. 4. Analysis of antitumor effects.
(A) Relative growth curves of tumors in four treatment groups: control, CA4P (30 mg/kg), VEC NAb (40 μg), combination of CA4P (30 mg/kg) and VEC NAb (40 μg). The relative tumor volume after transplantation was calculated by the formula: tumor volume each day/tumor volume at day 12. VEC NAb was administered on days 12 and 15. CA4P was administered 21 h after VEC NAb treatment (days 13 and 16). Error bars represents means ± SD for n = 5. P < .05 was considered significant, Tukey's test, *P < .05, **P < .01. (B) 95% family-wise confidence level (the 95% confidence interval of the differences in the mean value of relative tumor volume at day 20 after tumor transplantation, between groups in this study). The different treatment groups were: 1, control; 2, CA4P (30 mg/kg); 3, VEC NAb (40 μg); and 4, combination group (30 mg/kg CA4P and 40 μg VEC NAb).

microscopic metastasis of HMPOS cells was found 4 weeks after transplantation. In this study, macroscopic metastasis was not observed in the all normal tissues (data not shown). In addition, all tumor xenografted mice were euthanized within 3 weeks. Although the possibility of micro metastasis cannot be denied, it is presumed that it was very small amount. Local involvement to other organs or tissues, such as bone around the tumor was not confirmed. Although it was not compared with other tumor cell lines, we considered that HMPOS was useful cell line for tumor vascular research because of high micro blood vessel density in the tumor tissue.

It was reported that the area under the blood concentration-time curve for mice administered 25 mg/kg CA4P was similar to that found after CA4P treatment of humans at the maximum tolerated dose (Rustin et al., 2003; Tozer et al., 2002). The maximum tolerated dose of CA4P in humans is 60–68 mg/m^2, and is comparable to that for dogs (75 mg/m^2) (Abma et al., 2017b; He et al., 2011). In this study, only a partial blood flow blocking effect was observed with the administration of 30 mg/kg CA4P, which was assumed to be higher than the maximum tolerated dose in humans. These results indicate that, at the maximum tolerated dose in humans, CA4P might not be able to sufficiently block blood flow in tumors. In fact, a study, in which CA4P was administered at 68 mg/m^2 to human cancer patients and changes in tumor blood flow were observed using DCE-MRI, reported that K^{trans}, an indicator of changes in blood flow, decreased by only about 30% at most (Gaya et al., 2008). However, it is possible that administering a drug to inhibit VE-cadherin, in combination with CA4P at the maximum tolerated dose, might cause sufficient blood flow blockage to exert antitumor effects in both humans and dogs.

The greatest suppression of tumor growth found in this study was induced by the combination treatment, in which the relative rate of increase was significantly lower than in the control group. As the tumor sizes varied considerably at the start of treatment (length range: 2–8.5 mm), the antitumor effect was estimated based on changes in relative tumor size. However, even in humans or companion animals with naturally occurring tumors, evaluation of the effectiveness of treatment is made on the basis of relative changes in tumor size (Eisenhauer et al., 2009; Nguyen et al., 2015). Therefore, although the method used in this study is not generally applied in mouse experiments, it was considered to be an appropriate approach for estimating antitumor effects in tumor-xenografted mice. Previously, it was shown that a combination of CA4P and VE-cadherin neutralizing antibody could inhibit tumor growth and vascular density in another tumor model (Vincent et al., 2005). That report focused on the antiangiogenic effect of a combination therapy. By contrast, the present study focused on the tumor blood flow blocking effect of combination therapy. The novelty of this study is its focus on this aspect of the consequences of treatment. This study together with past reports show that combination therapy not only enhances the blood flow blocking effect in tumors, but also enhances the antiangiogenic effect even though further experiment is required to elucidate whether vascular blockade is a direct cause of antitumor effect. The overall antitumor activity might be enhanced by these two effects.

A second limitation of this study was that a non-immune IgG control group was not included; therefore, it is possible that a nonspecific antibody reaction affected the experimental results, such as the blood flow blocking effect and antitumor effect. However, a previous study reported that a non-immune IgG antibody did not affect tumor growth (Corada et al., 2002). In addition, in this study, single administration of VE-cadherin neutralizing antibody did not affect tumor blood flow, which indicated that a nonspecific antibody reaction was unlikely to have influenced tumor blood flow. Hence, we consider that the possibility of a nonspecific antibody reaction on the results of this study is low.

In conclusion, the selective tumor vascular blocking effects of CA4P were enhanced by the concomitant use of VEC NAb in canine osteosarcoma-xenografted mice. This study strongly suggests a relationship between the mechanism underlying selective blood flow blockage by CA4P and the expression of VE-cadherin in tumor blood vessels. Further experimentation is required to elucidate the abnormalities in VE-cadherin in tumor blood vessels, and these experiments should provide valuable insights for the development of more effective anti-tumor vascular therapies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2018.10.017.

Acknowledgments

The authors would like to thank S. Konnai and N. Maekawa (Laboratory of Infectious Disease, Hokkaido University) for helpful comments and technical advice.

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