Research in Veterinary Science 112 (2017) 222–228

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Effects of combretastatin A-4 phosphate on canine normal and tumor tissue-derived endothelial cells

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ARTICLE INFO

Keywords:
Combretastatin A-4 phosphate
Endothelial cells
Canine
Tumor
VE-cadherin

ABSTRACT

Combretastatin A-4 phosphate (CA4P) selectively blocks tumor blood flow. However, the detailed mechanisms through which CA4P specifically affects tumor blood vessels are not well understood. Recent reports revealed that tumor tissue-derived endothelial cells (TECs) have various specific features in comparison with normal tissue-derived endothelial cells (NECs). Thus, abnormalities in TECs may be involved in the selective vascular blockade mechanism of CA4P. In this study, we evaluated the effects of CA4P on canine NECs and TECs using confocal microscopy. NECs exhibited different susceptibilities at subconfluence and at 100% confluence. In addition, inhibition of vascular endothelial cadherin (VE-cadherin) in NECs increased the sensitivity of the cells to CA4P. TECs seemed to be more susceptible to CA4P than NECs. The expression pattern of VE-cadherin in TECs was abnormal compared with that of NECs, suggesting that VE-cadherin may have functional abnormalities in these cells. Taken together, these results indicate that the tumor-vascular selectivity of CA4P may be related to VE-cadherin dysfunction in TECs.

1. Introduction

Tumor vasculature, which supplies nutrients and oxygen to tumor cells, is necessary for tumor growth and metastasis. Antivascular therapy inhibits blood supply to cancer tissues, resulting in tumor necrosis. Several antivascular drugs have been developed in both human and veterinary medicine, including bevacizumab, sunitinib, and toceranib (Lin et al. 2016; Ranieri et al. 2013). This attractive therapeutic strategy can lead to prolonged survival in human patients with cancer who do not respond to conventional anticancer drugs.

Combretastatin A-4 phosphate (CA4P), derived from the African tree *Combretum caffrum*, is classified as a vascular disrupting agent (VDA). VDAs selectively block blood flow to the tumor within a few hours, causing tumor necrosis *in vivo* (Liang et al. 2016). Interestingly, VDAs do not disrupt normal blood vessels (Salmon et al. 2007). CA4P inhibits tubulin polymerization by binding to the colchicine-binding site after being dephosphorylated and converted to combretastatin A-4 (Tozer et al. 2005). CA4P also causes actin cytoskeletal reorganization and cell contraction through the small GTPase Rho and Rho kinase (Rho/ROCK) pathway in cultured endothelial cells (Kanthou and Tozer 2002). Researchers have proposed that contracted endothelial cells and increasing vascular permeability induced by CA4P may inhibit blood flow directly by narrowing the vascular lumen (Tozer et al. 2005). However, this hypothesis cannot explain the tumor vasculature-specific targeting of CA4P. Endothelial cell-cell junctions in the tumor vasculature are relatively weak compared with those of normal vasculature (Dudley 2012). As intercellular adhesion stabilizes cell morphology (Jamora and Fuchs 2002), the state of endothelial cell junctions may play a key role in determining the vascular selectivity of CA4P.

Recent reports have revealed that tumor tissue-derived endothelial cells (TECs) have abnormal characteristics, such as overexpression of specific markers, cytogenetic abnormalities and drug resistance, compared with normal tissue-derived endothelial cells (NECs) (Hida et al. 2004; Ohga et al. 2012). Specific features of TECs may increase susceptibility to CA4P. Therefore, a comparison of the sensitivities of TECs and NECs to CA4P may be essential for elucidating the mechanisms through which CA4P induces selective vascular shutdown.

Despite the need for further development of antivascular therapies in the veterinary field, few reports have investigated the effects of CA4P on companion animals *in vitro* and *in vivo*. Experiments using canine NECs and TECs will provide fundamental knowledge of the effects of CA4P in tumor-bearing dogs. Accordingly, in this study, we aimed to investigate the relationship between endothelial cell-cell junctions and sensitivity to CA4P using canine NECs and TECs.

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http://dx.doi.org/10.1016/j.rvsc.2017.05.017
Received 28 February 2017; Received in revised form 19 April 2017; Accepted 12 May 2017

2. Materials and methods

2.1. Isolation and culture of endothelial cells

All sample collection from dogs was performed according to the guidelines of Hokkaido University. No animal was sacrificed during this study. TECs were isolated from two dogs with spontaneous tumors, one with thyroid carcinoma (TC) and one with perianal gland epithelioma (PGE). Canine tissues from the two cases were surgically resected for the purpose of treatment, and informed consent was obtained from all owners pre-operatively at the Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University in Sapporo city in 2016. The dogs were randomly chosen and did not undergo any procedures related to this study. Histological diagnosis was performed by a board-certified veterinary pathologist. Tumor tissues were minced and digested using collagenase D (Roche Diagnostics, Mannheim, Germany). Digested cells were filtered through a 100-μm nylon mesh (Kyosin Riko, Tokyo, Japan) to remove undigested tissues. Then, filtered cells were cultured on collagen-coated dishes (Corning, Corning, NY, USA) in EGM2-MV (Takara Bio, Shiga, Japan) containing 5% fetal bovine serum (FBS) at 37 °C with 5% CO_2 in a humidified atmosphere. The dishes were washed with phosphate-buffered saline (PBS) 1 h later to remove unattached and weakly attached cells, such as red blood cells, white blood cells, and fibroblasts, and then further incubated in EGM2-MV for 3 days. Endothelial cells were purified using a magnetic cell sorter (EasySep magnet; StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Briefly, cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) and blocked with 10% goat serum in PBS. Cells were then stained with bispecific tetrameric antibody complexes (TACs), which recognize CD31-positive cells and dextran. These complexes were created using mouse IgG1 anti-CD31 monoclonal antibodies (clone: JC70A; DAKO, Glostrup, Denmark), which target CD31, a specific marker of endothelial cells, and a “Do it yourself” Selection Kit (StemCell Technologies) according to the manufacturer's instructions. TAC-labeled cells were further reacted with dextran-coated magnetic nanoparticles (EasySep Magnetic Nanoparticles; StemCell Technologies). Cells were suspended in a total volume of 2.5 mL in a 12 × 75 mm Falcon tube (Falcon Labware, Oxnard, CA, USA) by adding the buffer containing 2% FBS and 1 mM EDTA in PBS. Magnetically labeled cells were isolated using an EasySep magnet. Isolated cells were cultured in EGM2-MV. Medium was changed every 48 h.

NECs were isolated from the jugular vein of a healthy dog after euthanization. The dog was sacrificed for the purpose of another study, unrelated to this research, and this previous study was approved by the Institutional Animal Care and Use Committee of the National University Corporation, Hokkaido University. The jugular vein was cut along the major axis and cultured in EGM2-MV on a collagen-coated dish such that the vascular lumen was in the face-down position. Tissue was removed after 3 days of culture. After reaching subconfluence, NECs were purified using an EasySep magnet.

If contamination of other cells was observed, immunomagnetic purification of endothelial cells was performed again. The purity of the isolated cells was examined using flow cytometry.

2.2. Flow cytometry

Cultured NECs and TECs were detached from dishes using a scraper and incubated with 10% goat serum in PBS for 30 min on ice. Cells were stained with mouse IgG1 anti-CD31 monoclonal antibodies (1:100 dilution; DAKO), which have been reported to react with canine endothelial cells (Ferrer et al. 1995), or fluorescein isothiocyanate (FITC)-conjugated isolectin B4 (1:100 dilution; Vector Labs, Burlingame, CA, USA) for 30 min at 4 °C. The cells were also stained with mouse IgG1 isotype control (1:100 dilution; DAKO). After washing, the stained cells were further incubated with phycoerythrin (PE)-conjugated anti-mouse IgG antibodies (1:300 dilution; Abcam, Cambridge, MA, USA) for 30 min on ice. Cells were washed three times and analyzed using a FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.3. Assessment of the relationship between intercellular junctions and sensitivity to CA4P

CA4P was purchased from Selleck Chemicals (Houston, TX, USA). NECs were cultured until 100% confluent or 70–80% confluent (subconfluent). Cells were incubated with CA4P (100 nM or 1 μM) for 30 min or 3 h and then fixed in 4% paraformaldehyde for 10 min and observed by confocal microscopy. Additionally, 100% confluent NECs were also incubated under the following conditions: (i) ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; 4 mM; Nacalai Tesque, Kyoto, Japan) in EGM2-MV was added for 30 min before incubation with CA4P (1 μM, 3 h) to inhibit calcium-dependent intercellular junctions; and (ii) Y27632 (10 μM; Wako, Osaka, Japan) was added for 30 min after incubation with EGTA (4 mM, 30 min) to inhibit the Rho/ROCK pathway, and cells were then incubated with CA4P (1 μM, 3 h). TECs were cultured until reaching 100% confluent, and CA4P (1 μM, 3 h) was then added.

2.4. Effects of tumor conditioned medium on NECs

Tumor conditioned medium was obtained from HMPOS canine osteosarcoma cells (Barroga et al. 1999). HMPOS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10% FBS until reaching subconfluence, the medium was changed, and cells were further incubated for 24 h. The medium was collected and centrifuged to avoid cell contamination. The supernatant was then moved to another tube and stored at −20 °C until use. At 100% confluence, NECs were cultured with the tumor conditioned medium for 24 h, and CA4P (1 μM, 3 h) was then added. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

2.5. Immunofluorescence analysis

Cells were cultured on collagen-coated chamber slides (Nitta Gelatin, Osaka, Japan), fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 10% goat serum in PBS for 1 h at room temperature.

Cytoskeletal analysis was performed with β-tubulin and actin staining. Cells were incubated with Alexa Fluor 488-conjugated anti-β-tubulin monoclonal antibodies (1:300 dilution; Abcam) overnight in the dark at 4 °C. Subsequently, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (25 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added for 50 min at room temperature to visualize actin under a confocal microscope.

Morphological analysis of intercellular junctions was performed by visualizing vascular endothelial cadherin (VE-cadherin). Cells were incubated with FITC-conjugated anti-VE-cadherin polyclonal antibodies (50 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight in the dark at 4 °C. Cells were then washed three times with PBS and mounted with ProLong Diamond Antifade mountant with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Life Technology, Gaithersburg, MD, USA). Immunofluorescence images were obtained by confocal microscopy (LSM 700; Zeiss, Oberkochen, Germany). All experiments were repeated at least three times to confirm reproducibility.

3. Results

3.1. Isolation of NECs and TECs

NECs were isolated from the jugular vein of a healthy dog. TECs