NOTE *Surgery*

Isolation and characterization of canine tumor endothelial cells

Yusuke IZUMI[1)], Yuki HOSHINO[2)]*, Kenji HOSOYA[1)], Satoshi TAKAGI[2)] and Masahiro OKUMURA[1)]

[1)]*Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060–0818, Japan*
[2)]*Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060–0818, Japan*

(Received 2 July 2014/Accepted 16 November 2014/Published online in J-STAGE 5 December 2014)

ABSTRACT. The present study involved the isolation and characterization of canine tumor endothelial cells (TECs) from 2 malignancies. TECs were isolated using magnetic cell sorting following FITC labeling with UEA1 lectin, and they were characterized by measuring genetic and histopathological endothelial markers. Isolated TECs exhibited a cobblestone-like morphology and expressed both vascular endothelial growth factor receptor 2 (VEGFR2) and Von Willebrand factor (vWF). Further, both TECs and tumor cells derived from a seminoma exhibited increased C-X-C chemokine receptor type 7 (CXCR7) expression. However, CXCR7 expression was not detected in TECs and tumor cells derived from a hepatocellular carcinoma. Understanding TEC specific traits may be important in the development of more efficacious anti-angiogenic therapies that do not induce adverse effects.
KEY WORDS: angiogenesis, canine, tumor endothelial cell

doi: 10.1292/jvms.14-0347; *J. Vet. Med. Sci.* 77(3): 359–363, 2015

Anti-angiogenic therapies function by inhibiting tumor angiogenesis. Several anti-angiogenic therapies have been developed in veterinary medicine, including bevacizumab, toceranib and metronomic chemotherapy [10]. Conventional anti-angiogenic drugs target not only tumor endothelial cells (TECs), but also normal endothelial cells (NECs), which may cause adverse effects, including disruption of general perfusion, hypertension, hemorrhage and delayed wound healing [5].

Recently, a few reports indicated that the morphology and genetic expression of human and mouse TECs might differ from NECs [4, 12]. Understanding the characteristics of TECs is essential to the establishment of a new generation of anti-angiogenic therapies with fewer adverse effects [2]. The present study involved the isolation and comparison of the characteristics of canine TECs derived from a hepatocellular carcinoma (HCC) and a seminoma that had metastasized to sublumbar lymph nodes.

Canine tissues from 2 cases of surgically resected spontaneous tumors diagnosed clinically were evaluated. Informed consent was obtained from all owners preoperatively. TECs were isolated from HCC and seminoma specimens, and to serve as a control, NECs were isolated from subcutaneous adipose tissue or falciform ligament of liver. Excised tissues were minced and digested with collagenase II (Wako, Osaka, Japan), and undigested tissue was removed by filtration through a 100-μm nylon mesh (Kyoshin Riko, Tokyo, Japan). Blood cells were removed by a specific gravity centrifugal method using LymphoPrep (Cosmo Bio, Tokyo, Japan). The endothelial cells were then isolated using a magnetic cell sorting system (EasySep, STEMCELL Technologies, Vancouver, Canada) following FITC labeling with Ulex europaeus agglutinin-1 (UEA1) lectin, a canine endothelial marker (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.), coupled to magnetic beads [6]. Isolated cells were cultured using the EGM2-BulletKit (Takara Bio, Otsu, Japan) on gelatin-coated plates with 10% fetal bovine serum (Biowest, Miami, FL, U.S.A.).

RNA was extracted from the isolated cells using TRIzol (GIBCO BRL, Gaithersburg, NY, U.S.A.), and cDNA was synthesized using the TaKaRa RNA PCR Kit (Takara Bio) according to the manufacturer's protocol. The PCR thermal cycle conditions were as follows: initial step at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 10 sec. PCR products were electrophoresed onto a 2% agarose gel (Gene Pure LE, Biomedical science, Tokyo, Japan) containing ethidium bromide.

Quantitative RT-PCR was performed using Rotor-Gene Q (Qiagen, Hilden, Germany) with the KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Boston, MA, U.S.A.) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences are provided in Table 1.

Isolated cells were detached with 2 mg/m*l* EDTA and stained with one of two endothelial markers, FITC-conjugated mouse anti-αVβ3 integrin monoclonal antibody (Merck Millipore, Billerica, MA, U.S.A.) or FITC-conjugated Griffonia simplicifolia 1 isolectin B4 (GS1-B4, Vector Labs, Burlingame, CA, U.S.A.) for 30 min at 22°C. A minimum of 10,000 cells per sample were analyzed on a BD FACSverse™ flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, U.S.A.).

*CORRESPONDENCE TO: HOSHINO, Y., Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 jo Nishi-9 cho-me, kita-ku, Sapporo, Hokkaido 060–0818, Japan. e-mail: yhoshino@vetmed.hokudai.ac.jp

Table 1. Target primers used in this study

Target Gene	Amplication Size (bp)	Sequences	Genebank No.
VEGFR2	166	forward 5′- CTGGAGCCTACAAGTGCTTCTATCGG-3′ reverse 5′-GACCCAAGACATGGAA TCACCACAG-3′	NM_001048024
vWF	158	forward 5′-ATTCAGCTAAGAGGAGGAC-3′ reverse 5′-GCCAGACACTTGTGTTCATC-3′	NM_001002932
MDR1	95	forward 5′-ACTCGGGAGCAGAAGTTTGA-3′ reverse 5′-AATGAGACCCCGAAGATGTG-3′	NM_001003215
TEM8	160	forward 5′-AAGGCGCTAAGTTGGAAAAGGC-3′ reverse 5′-ACCCACAAGGCATCCAGTTTTC-3′	XM_850334
CXCR7	179	forward 5′-ACCTACTGCCGCTCCTTCTA-3′ reverse 5′-ATCTTTCGGCTGCTCTGCTT-3′	NM_020311
GAPDH	129	forward 5′-CTGAACGGGAAGCTCACTGG-3′ reverse 5′-CGATGCCTGCTTCACTACCT-3′	XM_003435649

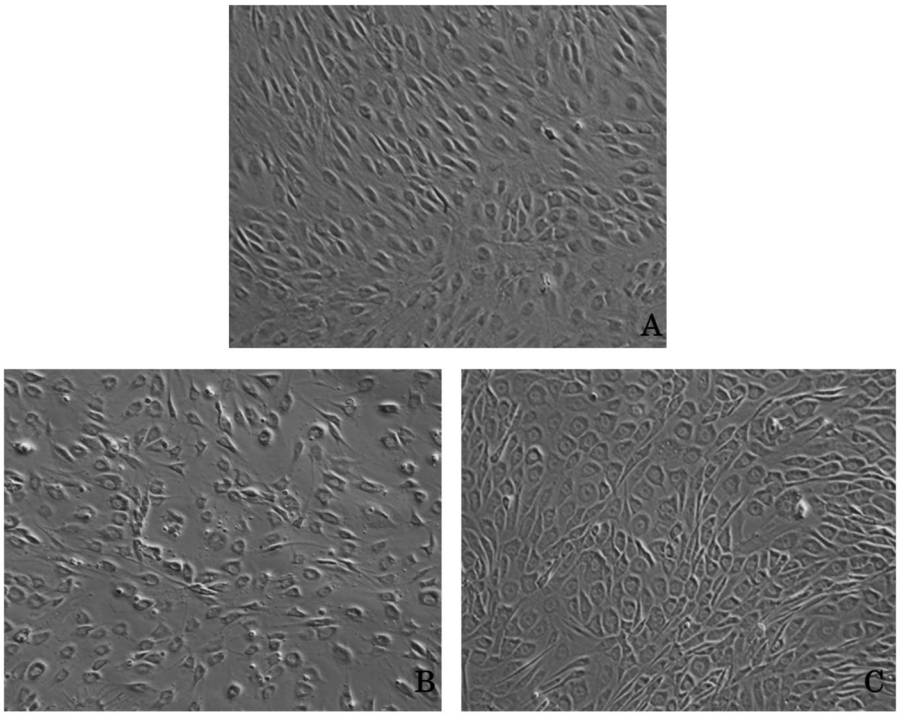

Fig. 1. Phase contrast microphotographs of the isolated cells obtained from (A) adipose tissues, (B) HCC and (C) Seminoma (Magnification, ×100). All isolated cells showed a cobblestone morphology, suggested endothelial cells.

The HCC and seminoma tissue samples were embedded in paraffin, cut into 5-μm sections and labeled with FITC-conjugated GS1-B4 and rabbit polyclonal anti-C-X-C chemokine receptor type 7 (CXCR7) primary antibodies (Abcam, Cambridge, MA, U.S.A.). Alexa Fluor 555 anti-rabbit IgG (Life technologies, Grand Island, NY, U.S.A.) was used as the secondary antibody. The samples were visualized using a LSM700 Confocal Laser Scanning Microscope (ZEISS, Oberkochen, Germany). All statistical analyses were performed using a Student's *t*-test, and a $P<0.05$ was considered statistically significant.

The cells isolated from HCC, seminoma and adipose tissues all exhibited a cobblestone morphology (Fig. 1). Based on flow cytometry results, the expression of GS1-B4 and αVβ3-integrin indicated high purity of the isolated endothelial cells (Fig. 2). The RT-PCR results revealed the expression of the endothelial markers, vascular endothelial growth factor receptor 2 (VEGFR2) and Von Willebrand factor (vWF) in both TECs and NECs (data not shown), which confirmed that the isolated cells expressed endothelial cell-

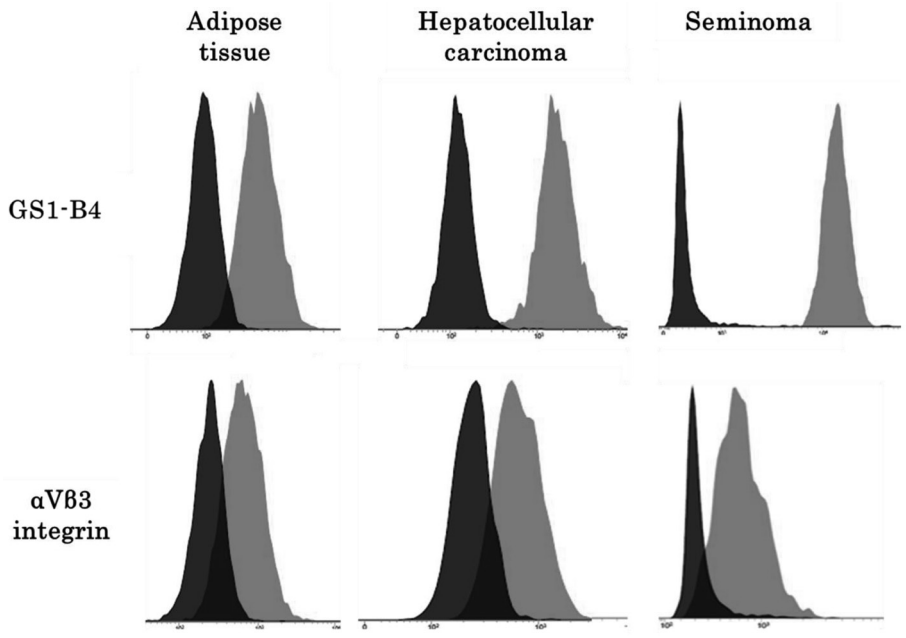

Fig. 2. Flow cytometric analysis. The isotype control was shown as a black area. GS1-B4 and αVβ3-integrin expression indicated high purity of isolated NECs and TECs.

specific features.

The expression of VEGFR2, multi-drug resistance (MDR1), tumor endothelial marker 8 (TEM8) and CXCR7 in the HCC and seminoma derived TEC populations was analyzed by quantitative RT-PCR. As well, the expression of the CXCR7 protein was evaluated using immunohistochemistry, and the results were compared with expression levels in NECs.

HCC derived TECs had significantly higher VEGFR2, MDR1 and TEM8 genetic expression than expression levels observed in NECs (data not shown). However, the expression of CXCR7 was not significantly different in TECs versus NECs (data not shown). In addition, TECs and tumor cells did not show expression of the CXCR7 protein in the histopathological specimens. (Fig. 3).

Seminoma derived TECs had significantly lower expression levels of VEGFR2, MDR1 and CXCR7, but higher TEM8 genetic expression than respective levels observed in NECs (data not shown). Histopathology revealed that CXCR7 protein expression was significantly higher in the TECs and tumor cells (Fig. 4).

All isolated cells exhibited characteristics as well as gene and protein expression patterns that were unique to endothelial cells and that indicated whether the isolated cells were TECs or NECs.

Several studies have reported increased expression of VEGFR2, MDR1, TEM8 and CXCR7 in human and mouse TECs when compared to NECs [1, 7, 8]. VEGFR2 is a primary signal transducer that is involved in tumor-associated angiogenesis [13]. The MDR1 gene produces a P-glycoprotein membrane-bound efflux pump against numerous anticancer drugs, which causes drug resistance in tumors [11]. TEM8 is a highly conserved cell-surface protein of unknown physiological function that is overexpressed in both tumors and tumor-infiltrating vasculature [3]. CXCR7 plays a critical role in tumor angiogenesis, as well as in the promotion of tumor development and progression [9].

Seminoma-derived TECs and tumor cells exhibited increased CXCR7 protein expression in histopathological specimens when compared to NECs. However, increased CXCR7 protein expression was not observed in HCC-derived TECs or tumor cells. CXCR7 is expressed on both human TECs and tumor cells, and its expression is correlated with tumor invasion and metastasis [14]. The HCC observed in this study was low malignant type that hardly metasized, while the seminomas were highly malignant and readily metastasized to lymph nodes. Therefore, the increased expression of CXCR7 in TECs might be correlated to tumor malignancy. In this study, the two malignant TEC tumors were investigated and cultured for a short time (under passage 4), and thus, further studies are required to further characterize canine TECs.

In conclusion, the characteristics exhibited by canine TECs isolated from HCC and seminomas were different from those of NECs. As well, our results indicated that differences may also exist between TECs derived from high and low malignancy tumors. Understanding TEC specific traits is important in the development of more effective anti-angiogenic therapies that do not induce adverse effects.

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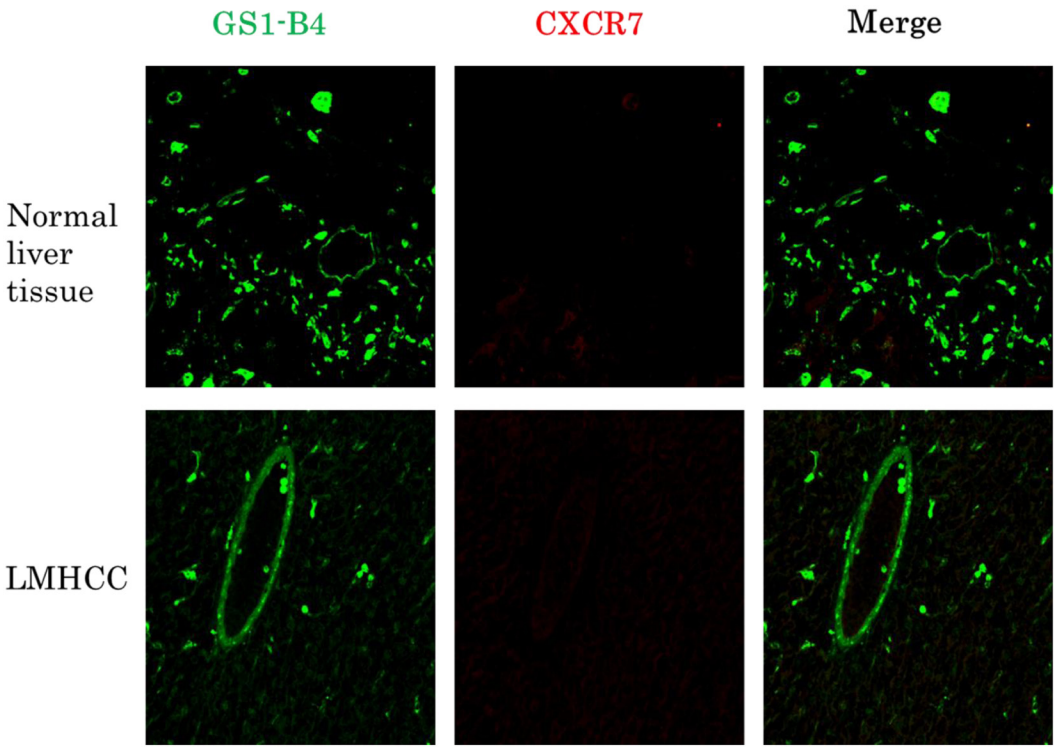

Fig. 3. Immunofluorescent double staining using GS1-B4 lectin and anti-CXCR7 antibody (Magnification, ×40). Tumor blood vessels in HCC and normal blood vessels in normal liver did not show expression of the CXCR7 protein.

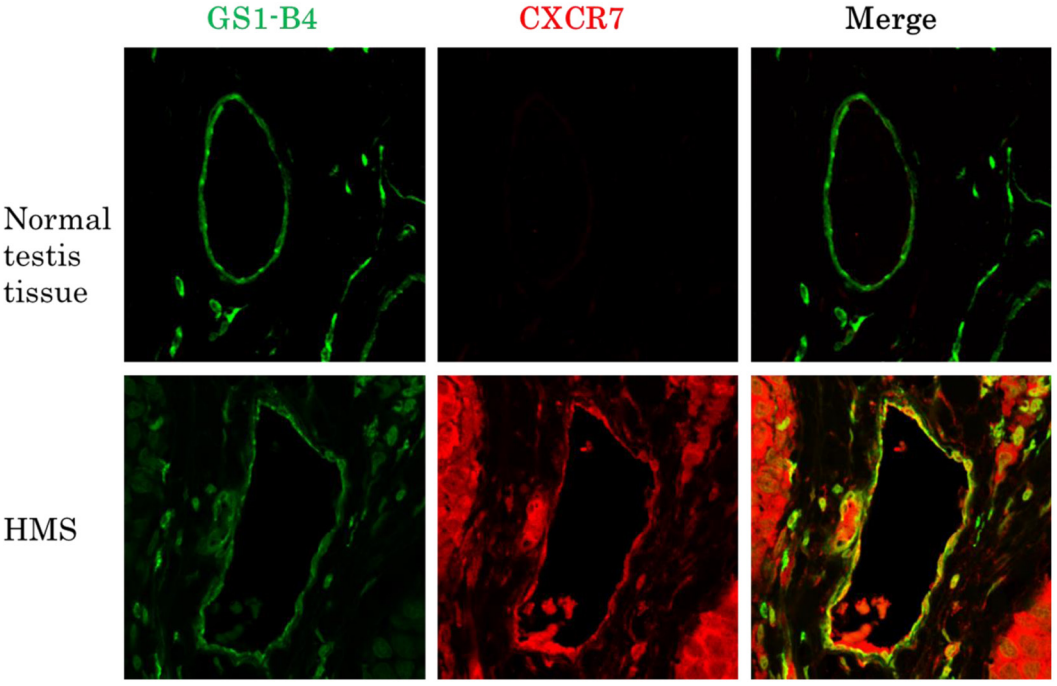

Fig. 4. Immunofluorescent double staining using GS1-B4 lectin and anti-CXCR7 antibody (Magnification, ×40). Tumor blood vessels and tumor cells in Seminoma showed significantly higher expression of CXCR7 protein, although normal blood vessels in normal testis did not show higher expression of the CXCR7 protein.

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