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Regulation of vascular endothelial growth factor (VEGF)-C and VEGF-D expression by the organ microenvironment in human colon carcinoma

Seiji Onogawa [a], Yasuhiko Kitadai [a,*], Shinji Tanaka [b], Toshio Kuwai [a], Tsuyoshi Kuroda [a], Kazuaki Chayama [a]

[a] *Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

[b] *Department of Endoscopy, Hiroshima University School of Medicine, Hiroshima, Japan*

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Abstract

Vascular endothelial growth factor (VEGF)-C and VEGF-D are potent lymphangiogenic factors produced by tumour and stromal cells. The purpose of this study was to investigate the expression of VEGF-C and VEGF-D in the organ microenvironment. We implanted human KM12 colon carcinoma cell lines into the subcutis and caecal wall of nude mice. The expression of VEGF-C and VEGF-D mRNAs and proteins were examined by reverse transcriptase–polymerase chain reaction and immunohistochemistry, respectively. Under culture conditions, VEGF-C mRNA was not detected in KM12 cells; however, VEGF-C expression was detected after implantation of KM12 cells into nude mice. VEGF-C and VEGF-D protein contents were higher in orthotopic (caecal wall) tumours than in ectopic (subcutis) tumours. Small vessels expressing VEGF receptor-3 were observed in the peripheral portions of caecal tumours. In metastatic liver tumours, VEGF-C and VEGF-D proteins were produced in lower amounts than those in caecal tumours. These data suggest that the expression of lymphangiogenic factors is influenced by the organ microenvironment. Therefore, experimental studies of colon cancer lymphangiogenesis should be performed with orthotopic implantation models.

Keywords: Colorectal carcinoma; Lymphangiogenesis; VEGF-C; VEGF-D; Organ microenvironment

1. Introduction

Cancer metastasis is a sequential and selective process that consists of a series of interlinked but independent steps, including angiogenesis, motility, invasion, adhesion, extravasation and proliferation [1]. Angiogenesis has been studied extensively, but lymphangiogenesis is a relatively new topic in vascular biology. Lymphangiogenesis and lymph-node metastasis are important for the spread of cancer. Lymph-node metastasis, a frequent occurrence in the early stages of many types of carcinoma, is considered a useful prognostic indicator [2]. Therefore, the control of lymph = node metastasis is an important consideration in cancer treatment.

Vascular endothelial growth factor (VEGF)-C and VEGF-D are ligands for VEGF receptor-3 (VEGFR-3/Flt-4), a tyrosine kinase receptor that is expressed predominantly in lymphatic endothelial cells [3]. VEGF-C and VEGF-D both stimulate growth of lymphatic vessels and were the first growth factors to be identified that were specific for lymphatic endothelial cells. Overexpression of VEGF-C and VEGF-D transgenes in experimental tumours revealed a direct correlation between lymphangiogenesis and lymph-node metastasis [4–6]. We previously reported that the expression of VEGF-C and VEGF-D correlates with lymph-node

* Corresponding author. Tel.: +81-82-257-5193; fax: +81-82-257-5194.
E-mail address: kitadai@hiroshima-u.ac.jp (Y. Kitadai).

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metastasis in colorectal carcinomas, and that VEGF-C and VEGF-D expression is heterogeneous and elevated at the invasive edge of tumours [7,8]. However, there have been few studies of the regulation of expression of lymphangiogenic factors.

In the present study, we investigated the regulation of expression of VEGF-C and VEGF-D in different organ microenvironments. KM12 colorectal carcinoma cells were implanted into the subcutis (ectopic site) or caecal wall (orthotopic site) of nude mice; the expression of VEGF-C and VEGF-D was found to vary in these different environments.

2. Materials and methods

2.1. Human colon carcinoma cell lines

Two human colon carcinoma cell lines, KM12C and KM12SM, were kindly provided by Dr. I.J. Fidler (University of Texas, Houston, TX, USA). The poorly metastatic KM12C cell line was originally isolated from a primary colon carcinoma classified as Dukes' stage B2 [9]. The highly metastatic KM12SM cell line was isolated from a liver metastasis produced by parental KM12C cells growing in the caecal wall of a nude mouse [9]. Cells were maintained in RPMI-1640 (Nissui Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS) (M.A. Bioproducts, Inc., Walkersville, MD, USA).

2.2. Animals and production of tumours

Male athymic BALB/c nude mice (6 weeks old) which originated in the Central Institute for Experimental Animals (Kawasaki, Japan) were purchased from CLEA Japan (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions. To produce tumours, cells growing in culture were harvested by brief treatment with 0.25% trypsin and 0.02% EDTA. A single-cell suspension of 1×10^6 cells with a viability of >95% was implanted into the subcutis or caecal wall of nude mice as described previously [9]. After 8 weeks, mice were killed, and tumours exceeding 6 mm in diameter were resected for study. The tumours were fixed in 10% buffered formalin for immunohistochemistry (IHC) or snap frozen for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

2.3. Semiquantitative RT-PCR analysis

Total RNA was extracted from cultured cells and tissues with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Primers were designed from synthesised for the reported sequences of human VEGF-C and were 5′-AGTTTTGCCAATCACACTTCCTG-3′ and 5′-GTCATTGGCAGAAAACCAGTCTT-3′ [10,11]. Primers for the *VEGF-D* gene were 5′-GTATGGACTCTCGCTCAGCAT-3′ and 5′-AGGCTCTCTTCATTGCAACAG-3′ [12]. RT-PCR was performed with the isolated RNA (1 μg). To validate the quantitative nature of our RT-PCR method, ethidium bromide stainings for specific mRNA bands were examined on the basis of various numbers of PCR cycles. The intensity of the VEGF-D PCR product was calculated with computer software (*Photoshop 6.0*, Adobe, USA; *NIH Image 1.63*, Wayne Resband, NIH, USA). The ethidium bromide stainings for VEGF-C and VEGF-D increased linearly up to 35 cycles [8]. VEGF-C cDNA was amplified with 35 cycles of denaturation for 2 min at 94 °C, annealing for 2 min at 55 °C and extension for 3 min at 72 °C. VEGF-D cDNA was amplified with 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C and extension for 1 min at 72 °C. After the amplification, the mixtures were separated by electrophoresis on 5% non-denaturing polyacrylamide gels in Tris-borate-EDTA buffer (TBE). RT-PCR reactions without the reverse transcriptase yielded no specific bands.

2.4. IHC

Consecutive 4-μm sections were cut from each study block. IHC for VEGF-C and VEGF-D was done with the immunoperoxidase technique following trypsinisation. Antibodies used were goat polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilutions for VEGF-C (N-19) and VEGF-D (N-19). Negative controls had non-specific IgG as the primary antibody. Immune complexes were visualised with the CSA system (Dako, Glostrup, Denmark). IHC reactions for VEGF-C and VEGF-D were scored with image analysis software (*Image-pro Plus*™ version 3.0.1; Media Cybernetics, L.P., Carlsbad, CA, USA). The ratio of the area of stained cells to the total area was calculated. Six different fields were quantified for each sample, and the average value was calculated.

IHC for VEGFR-3 was done with the immunoperoxidase technique following microwave treatment. We used an antimouse VEGFR-3 (Flt-4) rabbit polyclonal antibody (Alpha Diagnostics, San Antonio, TX, USA) at 1:100 dilution. Immune complexes were visualised with the LSAB system (Dako).

2.5. Statistical analyses

All statistical calculations were carried out with *StatView-J 5.0* statistical software (SAS Institute, USA). The significance of the differences in the intensities of staining for VEGF-C and VEGF-D between orthotopic and ectopic tumours was analysed by Student *t*-test. A value of $P < 0.05$ was deemed significant.

3. Results

3.1. Expression of VEGF-C and VEGF-D mRNAs in KM12 cells growing in vitro and in vivo

In the first set of experiments, we examined the expression of VEGF-C and VEGF-D mRNAs in KM12 cells growing in culture and in experimental tumour tissues by RT-PCR (Fig. 1). Neither cell line expressed VEGF-C mRNA under culture conditions, but expression of VEGF-C mRNA was observed when cell lines were implanted into nude mice. There was no obvious difference in VEGF-D expression between *in vitro* and *in vivo* conditions. The site of implantation did not affect the amounts of VEGF-C and VEGF-D mRNAs.

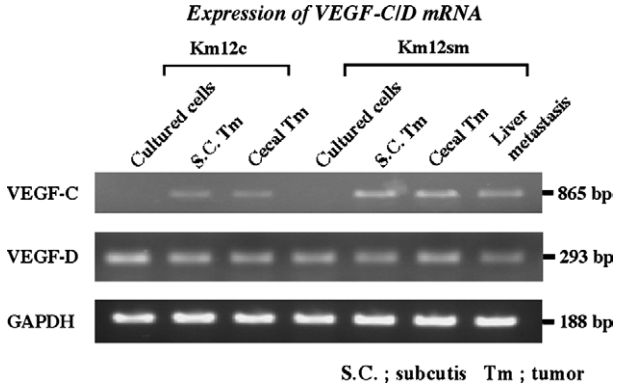

Fig. 1. Reverse transcriptase–polymerase chain reaction analysis of expression of vascular endothelial growth factor (VEGF)-C and VEGF-D mRNAs by KM12 cells growing as cultures or in nude mice. VEGF-C mRNA was not detected in cultured KM12 cells. GAPDH was included as an internal control. S.C., subcutis; Tm, tumour.

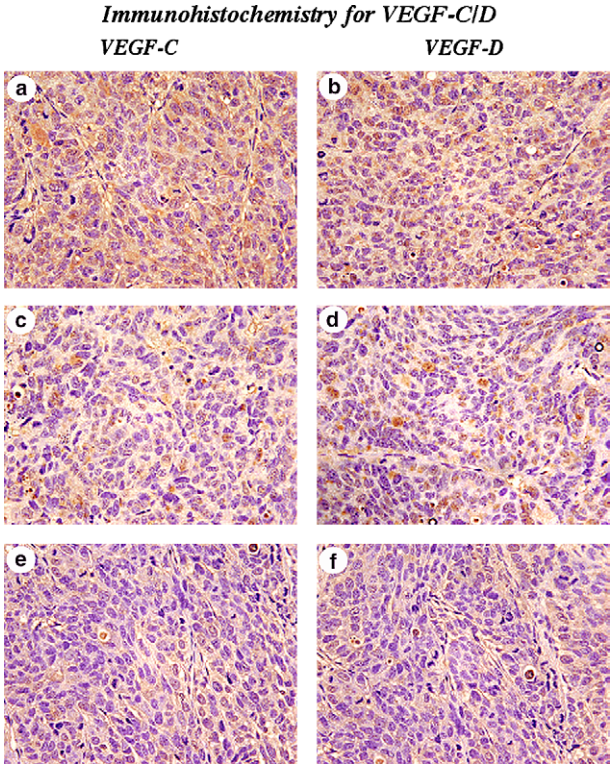

Fig. 2. Immunohistochemistry for vascular endothelial growth factor (VEGF)-C (a, c, e) and VEGF-D (b, d, f) in KM12 tumours. Expression of VEGF-C and VEGF-D was higher in caecal tumours (a, b) than in subcutis tumours (c, d). In metastatic lesions (e, f), VEGF-C and VEGF-D proteins were downregulated in comparison with caecal tumours.

3.2. Expression of VEGF-C, VEGF-D and VEGFR-3 proteins in caecal and subcutaneous KM12 tumours

In the second set of experiments, we used IHC to analyse KM12C (low metastatic potential) and KM12SM (high metastatic potential) cells growing in the caecum and subcutis of nude mice (Fig. 2). Moderate to strong immunoreactivities for VEGF-C and VEGF-D were observed in the cytoplasm of carcinoma cells (Fig. 2). Heterogeneous staining was observed. VEGF-C and VEGF-D expression was higher in peripheral parts of the tumour than in the central part. In both cell lines,

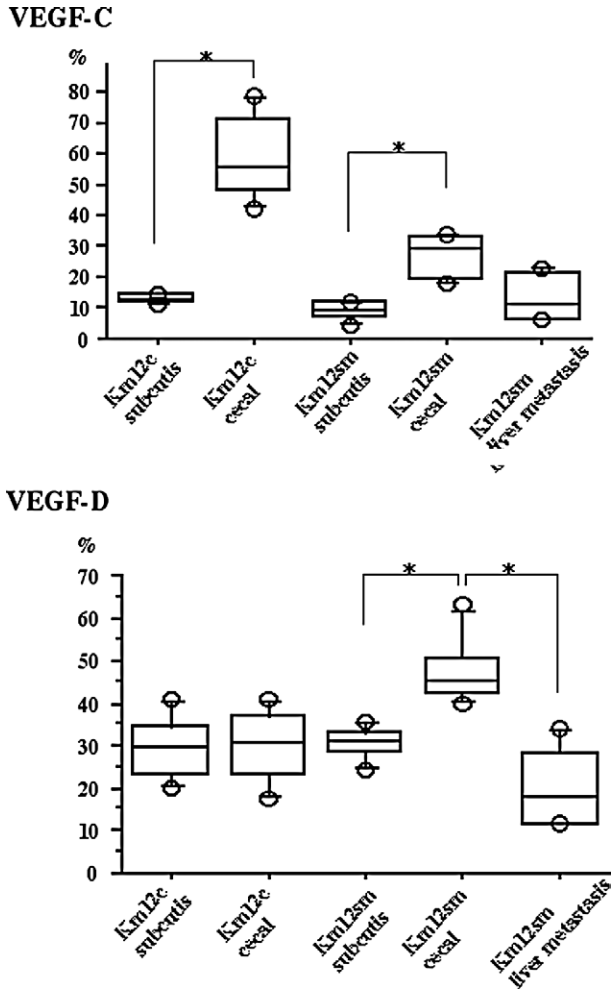

Fig. 3. Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D proteins in KM12 tumours growing in nude mice. $^{*}P < 0.05$.

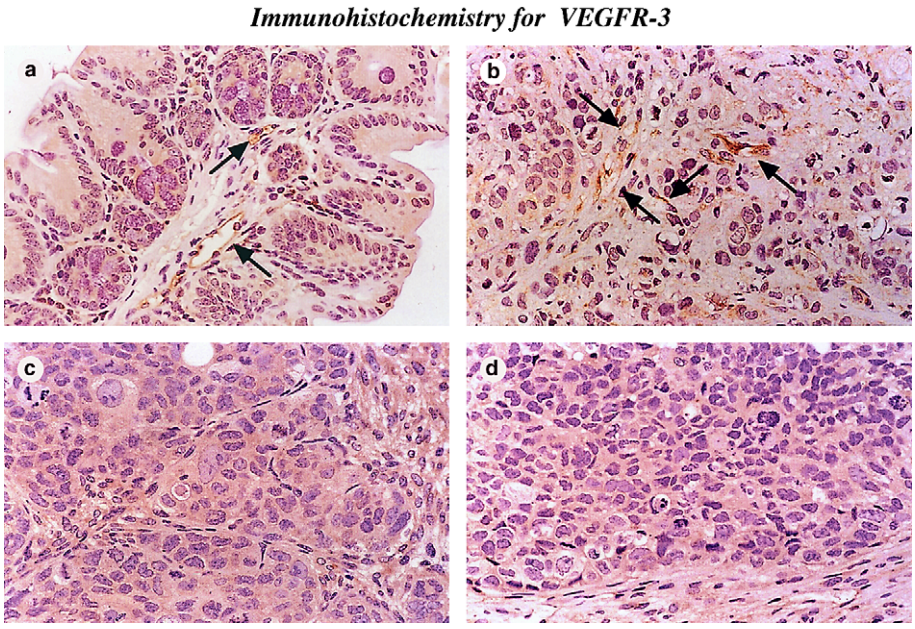

Fig. 4. Immunohistochemistry for vascular endothelial growth factor receptor (VEGFR)-3 in KM12SM tumours from nude mice. Lymphatic vessels in the submucosal layer were stained with VEGFR-3 (a). VEGFR-3-positive microvessels were increased in caecal tumours (b) but not in subcutaneous (c) or metastatic liver tumours (d).

expression of VEGF-C protein was higher in caecal than in subcutaneous tumours. The proportion of KM12C expressing VEGF-C was increased significantly from 12.6% in subcutaneous (ectopic) tumours to 58.6% in caecal (orthotopic) tumours, and the proportion of KM12SM cells expressing VEGF-C increased from 8.5% in subcutaneous tumours to 27.1% in caecal tumours, respectively. Moreover, expression of VEGF-D protein by KM12SM cells was increased from 30.7% in subcutaneous tumours to 47.6% in caecal tumours (Fig. 3). Liver metastasis was observed when KM12SM cells were injected into the caecal wall of nude mice. Contents of VEGF-C and VEGF-D proteins expressed by liver metastases were lower than those in caecal tumours (Fig. 3).

Lymphatic endothelial cells were stained with anti-VEGFR-3 antibody and revealed thin-walled vessels (Fig. 4(a)). In both KM12C cells and KM12SM cells, VEGFR-3-positive vessels were observed in the peritumoral lesion in caecal tumours but not subcutaneous or metastatic liver tumours (Fig. 4(b)–(d)). In the majority of tumours, lymphatic vessels appeared to be angulated or collapsed in intratumoral lesions.

4. Discussion

The influence of the organ microenvironment on the biology of tumour cells has been recognised since Paget's 'seed and soil' hypothesis, which suggested that the interactions between tumour cells and target organs determine whether metastasis will occur [13]. Organ-specific factors can influence growth, vascularisation, invasion and metastasis of human neoplasms. Morikawa and colleagues [9] showed that implantation of KM12 cells into the caecal wall (orthotopic) or subcutis (ectopic) of nude mice produces tumours with different metastatic potentials. Caecal (orthotopic) tumours are invasive and metastatic, whereas subcutaneous (ectopic) tumours are not, suggesting that different organ environments may differentially influence the expression of metastasis-related genes. We previously reported that the expression of mRNAs encoding epidermal growth factor receptor (EGFR) (growth), basic fibroblast growth factor (bFGF), interleukin 8 (angiogenesis) and type IV collagenase (invasion) is increased in caecal wall tumours in comparison with subcutaneous tumours [14]. However, the influence of the organ environment on lymphangiogenic factors has not been investigated.

In the present study, we examined the expression of VEGF-C and VEGF-D mRNAs and proteins *in vitro* and *in vivo*, and the influence of the organ microenvironment on the expression of these genes. We found that KM12 cells do not express VEGF-C mRNA under culture conditions, whereas implanted KM12 tumours do. We observed that orthotopic implantation of KM12C cells into nude mice induces high expression of VEGF-C protein in comparison with that in ectopic implantation. Highly metastatic KM12SM cells overexpress both VEGF-C and VEGF-D proteins after orthotopic implantation. It is unlikely that a clone expressing large amounts of these proteins grew selectively in the caecal wall because expression of VEGF-C and

VEGF-D was downregulated in the metastatic lesion (liver). These findings indicate that VEGF-C and VEGF-D expression by human colon carcinoma cells is influenced by the organ microenvironment.

In both cell lines, the number of vessels expressing VEGFR-3 was higher in the peritumoral lesion in caecal tumours than in subcutaneous tumours, but only KM12SM cells produce liver metastases. Because the metastatic process involves multiple steps including growth, angiogenesis, invasion, motility and adhesion, other metastasis-related factors may contribute to the different metastatic potentials between these cell lines. We previously found that expression of EGFR (growth), bFGF (angiogenesis), and CEA (adhesion) is higher in KM12SM cells than in KM12C cells both *in vitro* and *in vivo*.

RT-PCR analysis cannot reveal any site-dependent differential expression of VEGF-C and VEGF-D mRNAs (Fig. 1). Tumour tissues consist of multiple cell types including tumour cells and host, stromal, endothelial and infiltrating cells. RT-PCR of mRNAs isolated from bulk tissues represents the average amount of mRNAs for all the cells in the sample and cannot determine if a specific mRNA is derived from normal or tumour cells. RNA from a few contaminating cells may be amplified during RT-PCR and obscure tumour-specific alterations. In contrast, IHC can identify the cellular source as well as reveal intratumour heterogeneity in expression. Contamination of tumour tissues with mouse stromal cells may cause discrepant results between RT-PCR and IHC.

The molecular mechanism underlying the upregulation of VEGF-C and VEGF-D at the orthotopic site remains unclear. In fibroblasts, VEGF-C expression is induced by serum, phorbol 12-myristate 13-acetate, and factors such as interleukin 1β and tumour necrosis factor-α [15,16]. VEGF-D expression is induced in low-serum conditions and by cell–cell contacts mediated by cadherin-11 [17,18]. We previously reported that VEGF-D expression by colon carcinoma cells is upregulated under hypoxic conditions [8]. Schoppmann and colleagues [19] report that tumour cells and activated tumour-associated macrophages express VEGF-C and VEGF-D in human cervical carcinomas. The interactions between tumour cells and stromal cells may play a part in the regulation of lymphangiogenesis. Because VEGF-C and VEGF-D expression was higher at the periphery not the central part of the tumour, it is possible that the interaction of tumour cells and stromal cells is important. Further studies are needed to determine how the expression of lymphangiogenic factors in the colon is regulated.

In conclusion, the organ microenvironment influences the expression of lymphangiogenic factors. Although inhibitors of the VEGF-C/VEGF-D/VEGFR-3 signalling pathway might block lymphogenous metastatic spread, experimental studies of lymphangiogenesis in colon cancer should be done with orthotopic implantation models.

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