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Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer

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Abstract

Vascular endothelial growth factor C (VEGF-C) is the only factor known to cause lymphangiogenesis. We studied the correlation between *VEGF-C* and vascular endothelial growth factor receptor-3 (*VEGFR-3*) expression of 85 primary gastric cancers by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry, and the results were correlated with the number of lymphatic vessels, stained with anti-VEGFR-3 antibody. RT-PCR and immunohistology demonstrated that VEGF-C was mainly produced from cancer cells, but not from stromal elements. Morphologically, VEGFR-3 expression was detected in the endothelial cells of the stromal lymphatic vessels. There was a statistically positive correlation between the incidence of *VEGF-C* and *VEGFR-3* mRNA expression in the primary tumours ($P = 0.0002$). The number of VEGFR-3-positive lymphatic vessels in VEGF-C mRNA positive tumours was significantly larger than that in VEGF-C-negative tumours. The number of VEGFR-3-positive vessels in the tumour stroma was closely related to the grade of lymphatic invasion of gastric cancer. These results strongly indicate that VEGF-C may induce the proliferation of lymphatic vessels in the stroma of primary gastric cancer via activation of VEGFR-3, expressed on the endothelial cells of lymphatic vessels. In these circumstances, cancer cells can easily invade the lymphatic vessel, because of the increase of the contact points of cancer cells with the lymphatic vessels.

Keywords: VEGF-C; VEGFR-3; Gastric cancer; Flt4; Lymphangiogenesis

1. Introduction

Recent advances in molecular biology have clarified the mechanisms of the vasculogenesis, and this has been helped by the isolation of endothelial cell-specific growth factors and their signalling receptors. Vascular endothelial growth factor (VEGF) is well known as an important stimulator of vascular endothelial cell proliferation, migration and permeability and is upregulated in response to hypoxia [1,2].

Since the characterisation of VEGF, three other VEGF-related genes have been identified, including the placental growth factor (*PIGF*) [3], *VEGF-B* [4] and *VEGF-C* [5]. PIGF, which is 53% identical with VEGF, has a role in angiogenesis of the placenta [6]. These four growth factors competitively bind to their receptors, such as VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt-4) [7]. VEGF binds both VEGFR-1 and VEGFR-2 [8], and VEGF-C activates both VEGFR-2 and VEGFR-3 [9]. Activation of VEGFR-1 and VEGFR-2 results in the mitogenesis of the endothelial cells of blood vessels [5]. In contrast, VEGFR-3 activation by VEGF-C is considered to induce proliferation of lymphatic endothelial cells. From these results, VEGF-C is speculated to induce both angiogenesis and lymphangiogenesis [9], and VEGF-C is known as the only potential lymphoangiogenic factor [9].

During lymph node metastasis, proliferation and dilatation of lymphatic vessels are common findings in the stroma of the primary tumour. However, the molecular mechanism of the proliferation of lymphatic vessels

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in cancer tissues and lymph node metastasis are not understood.

The first step of lymph node metastasis is considered to be intravasation of the cancer cells into lymphatic vessels. Then, cancer cells migrate into the regional lymph nodes, and proliferate there to form a metastasis. Overexpression of growth factors [10,11], motility factors [12,13] and matrix digesting enzymes from cancer cells [13] are believed to be associated with the intravasation of cancer cells into the lymphatic vessels. Recently, we found that VEGF-C is involved in lymph node metastasis in gastric cancer [14].

We report herein the existence of a paracrine loop consisting of VEGF-C/VEGFR-3 in gastric cancer and its role in the proliferation of lymphatic vessels and the lymphatic system.

2. Materials and methods

2.1. Patients and tumour samples

85 patients with primary gastric cancer diagnosed and treated at the Second Department of Surgery II, Kanazawa University Hospital, between 1996 and 1998, were entered into the study.

Immediately after resection of the primary tumour, small pieces of tissue approximately 5 to 8 mm in diameter were cut from the primary tumour and adjacent normal mucosa and were fixed in acetone at −20°C overnight and then washed in methyl benzoate for 30 min and in xylane in a vacuum evaporating embedder (AMeX method) [22].

All the resected primary tumours and regional lymph nodes were histologically examined by haematoxylin-eosin staining according to the Japanese Classification of Gastric Carcinoma [15]. As positive controls for the mRNA and protein expression of VEGF-C and VEGFR-3, the prostate adenocarcinoma cell line (PC3) was used.

2.2. Antibody and immunohistochemistry

Anti-VEGF-C antibody (C-20) was purchased from Santa Cruz Biotech., Inc (San Diego, USA). C-20 is an affinity-purified goat polyclonal antibody raised against a peptide corresponding to amino acids 136–155 mapping at the carboxyl terminus of the VEGF-C precursor of human origin. Anti-VEGFR-3 mouse monoclonal antibody was a kind gift from K. Alitalo [5].

For immunohistochemical tests, paraffin sections 3–4 μm thick were deparaffinised and treated for 20 min with a blocking serum (normal rabbit serum for VEGF-C and normal goat serum for VEGFR-3) diluted 1:10. The sections were treated with anti-VEGF-C or anti-VEGFR-3 antibody diluted 1:100 in phosphate-buffered saline (PBS) and incubated at 4°C in a humidified chamber. Then, the slides were incubated with biotinylated rabbit anti-goat IgG for VEGF-C (×100) (Vectorstain ABC kits; Vector Laboratories) and with premixed avidin/biotin complex reagent (ABC) (Vector) reagent for 20 min. Slides for VEGFR-3 immunostaining were treated with the labelled streptoavidin-biotin (LSAB) kit (DAKO Laboratories). For immunostaining, both kinds of slides were treated with 0.06 mM diaminobenzidine (DAKO) and 2 mM hydrogen peroxide in 0.05% (w/v) PBS (pH 7.6) for 5 min.

2.3. Reverse transcription (RT)-polymerase chain reaction (PCR)

RT-PCR analysis was carried out according to the modifications by Conboy and colleagues [16]. Briefly, total RNA was extracted from each tissue using a RNA extraction reagent, Isogen (Nippon Gene, Tokyo), according to the standard acid-guanidium-phenol-chloroform method [17]. The prepared RNA was mixed with the oligo dT (50 pmol), incubated for 15 min at 68°C, and then quickly chilled in an ice bath for 5 min. The RNA samples were reverse-transcribed at 42°C for 60 min into first-strand cDNA in reverse transcriptase (RT) solution [16]. The cDNA samples were incubated at 95°C for 5 min to inactivate the reverse transcriptase, and then chilled. The samples were amplified by PCR [16]. The amplification conditions were 1.5 min at 94°C, 2 min at 48°C, 2 min at 72°C for three cycles, and then there were 25 cycles of 40 s at 94°C, 1.5 min at 48°C, 3 min at 72°C. The products of amplification were electrophoresed, and hybridised overnight to a [^{32}]-end-labelled probe specific for the target cDNA fragment.

Specific primers for the *VEGF-C* gene were VEC-S3; 5′- AGTTTTGCCAATCACACTTCCTG-3′ and VEC-A3: 5′-GTCATTGGCAGAAAACCAGTCTT-3′, the target fragment of which is 865 bp, and the probe oligonucleotide was VEC-P; 5′-GTCATGGAATCCATC-TGTTGAGT-3′. Primer sequences for VEGFR-3 were FLT4-1 (sense); 5′-AGCCATTCATCAACAAGCCT-3′, and FLT4-2 (antisense) (PCR product, 298 bp); 5′-GGCAACAGCTGGATGTCATA-3′, and the probe oligonucleotide (FLT4-A); 5′-AAACCTTGAAGTTG-CTGGTA-3′. Primers for *VEGF* were VEGF-1 (sense); 5′-GAAGTGGTGAAGTTCATGGATGTC-3′ and VEGF-2 (antisense) 5′-CGATCGTTCTGTATCAG-TCTTTCC-3′, and the target fragments are 408 bp (*VEGF121*), 541 bp (*VEGF165*) and 613 bp (*VEGF185*) [18]. The probe oligonucleotide for VEGF was VEGF-P; 5′-GAGATGAGCTTCCTACAGCACAAC-3′. Primer sequences for the *VEGFR-2* gene were 5′-TATA-GATGGTGTAACCCGGA-3′ (KDR-1, sense) and 5′-TTTGTCACTGAGACAGCTTGG-3′ (KDR-2, antisense), the target fragment of which is 555 bp, and the probe oligonucleotide was 5′-ATCCAGTGGGCT-GATGACCAAGAAGAACAG-3′ [18].

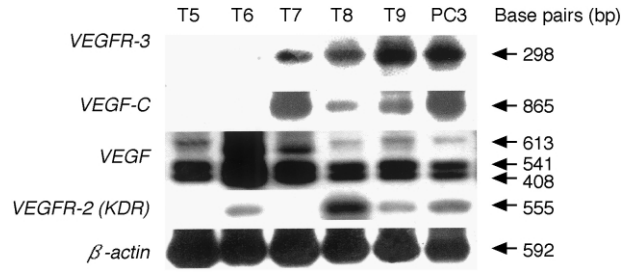

Fig. 1. Vascular endothelial growth factor receptor (*VEGFR-3*), *VEGFR-2* (KDR), *VEGF* and *VEGF-C* mRNA expression in primary gastric cancers and PC3 cells (reverse transcriptase-polymerase chain reaction (RT-PCR) method). T, tumour.

As an internal standard, primer pairs specific for the *β-actin* gene; β-act-5: 5′-TTGAAGGTAGTTTCGTGGAT-3′, β-act-11: 5′-GAAAATCTGGCACCACACCTT-3′, (PCR products: 592 bp) were used. β-act-7: 5′-ACTGACTACCTCATGAAGAT-3′ was used as a probe.

2.4. Western blotting

Twelve microlitres of protein sample (total protein 12 μg) was mixed with 6 μl sample buffer (50 mM Tris-HCl pH 6.5, 10% (w/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) bromophenol blue) and 0.6 μl dithiothrectol (DTT) prior to separation by the NuPAGE Electrophoresis System (NOVEX an Invitrogen Com., San Diego, USA). After completion of electrophoresis, samples were transferred to PVDF membrane filters (Immobilon, Millipore, Bedford, MA). The transferred samples were incubated with anti-VEGF-C and anti-VEGFR-3 antibody (1:2000) for 2 h. The membranes were incubated with the second antibody (horseradish peroxidase-conjugated antigoat IgG for VEGF-C and antimouse IgG, horseradish peroxidase linked F(ab')2 fragment for VEGFR-3) (1:5000, Santa Cruz Biotech., Lab., and Amersham Life Science) for 40 min, washed, and then incubated with the enhanced chemoluminescence (ECL) kit (Amersham Life Science Co., Ltd.) for several minutes. The membrane was then exposed for 10 min to develop the protein-specific signal.

2.5. Data presentation and statistical analysis

All statistical calculations were carried out using the Statistical Product and Services Solutions (SPSS) statistical software. Each figure is presented as the mean plus or minus the standard deviation of the mean (S.D.). The Chi-square test and Student's *t*-test was used to analyse data. Values with a P value of 0.05 or less were considered to be statistically significant.

Table 1
Association between tumours expressing *VEGF-C* and *VEGFR-3* mRNA in 85 primary gastric cancers

VEGF-C mRNA expression	*VEGFR-3* mRNA expression		
	Not detected	Detected	
Not detected	26 (68%)	12 (26%)	$P=0.0002$
Detected	12 (32%)	35 (74%)	

3. Results

3.1. VEGF-C, VEGFR-3, VEGFR-2 *and* VEGF *mRNA expression in primary gastric cancer (RT-PCR analysis)*

Primary gastric cancers and adjacent normal mucosa from the same patients were examined for the expression of *VEGF-C* and *VEGFR-3* mRNA by RT-PCR. *VEGF-C* was expressed in 47 of 85 primary tumours (55%) and 13% (11/85) of the normal tissue samples. *VEGFR-3* mRNA was expressed in 47 gastric cancers (55%) and 54 (64%) of the normal mucosa. *VEGFR-2* and *VEGF* mRNA was expressed in 46 (54%) and 62 (73%) of 85 primary tumours (Fig. 1).

Expression of *VEGF-C* and *VEGFR-3* in the primary tumours was significantly correlated ($P<0.05$) (Table 1). *VEGF-C* mRNA-negative tumours ($n=38$) showed *VEGFR-3* mRNA expression in only 12 (32%) tumours, but in the 47 *VEGF-C* mRNA-positive tumours *VEGFR-3* mRNA was coexpressed in 35 (74%) ($P=0.0002$). In addition, there was a statistically significant correlation between *VEGF-C* mRNA and *VEGFR-2* mRNA expression (Table 2). *VEGF-C*-producing tumours tended to express *VEGFR-2* mRNA ($P<0.05$).

Out of 62 *VEGF* mRNA-positive tumours, *VEGFR-2* mRNA was coexpressed in 42 (68%), but 23 VEGF mRNA-negative tumours expressed *VEGFR-2* mRNA in only 5 (22%). There was a statistical significant correlation between VEGF and VEGFR-2 mRNA expression ($P<0.05$). However, the tumours that expressed *VEGF* mRNA coexpressed VEGFR-3 in 36/62 (58%), and the 23 *VEGF* mRNA-negative tumours expressed

Table 2
Association between tumours expressing *VEGF-C* and *VEGFR-3* mRNA in 85 primary gastric cancers

VEGF-C mRNA expression	*VEGFR-2* (*KDR*) mRNA expression		
	Not detected	Detected	
Not detected	23 (59%)	15 (33%)	$P=0.027$
Detected	16 (41%)	31 (67%)	

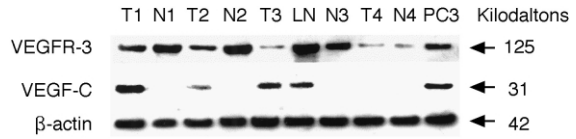

Fig. 2. VEGFR-3 and VEGF-C protein expression in primary gastric cancers and normal gastric mucosa (western blotting analysis). T, tumours; N, normal gastric epithelium; LN, lymph node metastasis.

VEGFR-3 mRNA in 11 (48%). No significant correlation was found in the expression pattern of *VEGF* and *VEGFR-3* mRNA in the primary tumours.

3.2. VEGF-C and VEGFR-3 protein expression in gastric cancer tissues and normal mucosa

C-20 and anti-VEGFR-3 antibodies recognised 31 and 125 kDa molecules, respectively (Fig. 2). The RT-PCR and western blotting results showed that VEGF-C was expressed mainly in gastric cancer tissues, but not in normal mucosa. Normal gastric epithelial cells did not show VEGF-C immunoreactivity. In contrast, western blot analysis showed that VEGFR-3 protein was expressed not only in the primary tumours, but also in normal mucosa. However, immunohistological study showed that VEGFR-3 immunoreactivity was restricted to the endothelial cells of mucosal or submucosal vessels devoid of red blood cells. VEGFR-3-positive vessels were mainly located in the lower half of normal gastric mucosa and in the submucosal tisssue, just beneath the muscularis mucosa. Endothelial cells of large blood vessels did not show VEGFR-3 immunoreactivity, but a very small number of endothelial cells in the small blood vessels expressed VEGFR-3. Accordingly, the majority of VEGFR-3-positive vessels were regarded as lymphatic vessels (data not shown).

In gastric cancer cells, VEGF-C was observed as diffuse cytoplasmic staining (Fig. 3). In the stroma of the primary tumours, many VEGFR-3-positive vessels with small diameters could be seen (Fig. 4). In the dilated VEGFR-3-positive vessels, cancer cell emboli were frequently found (Fig. 4).

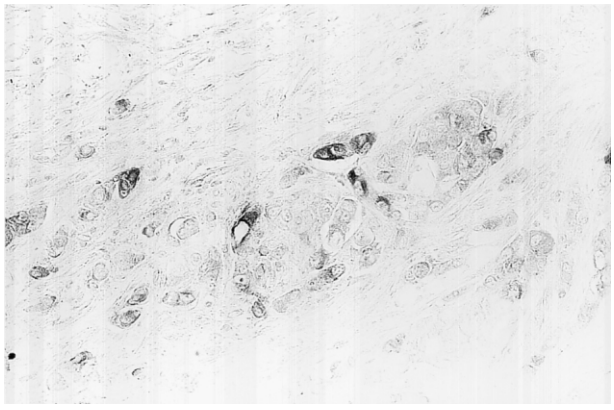

Fig. 3. VEGF-C-positive gastric cancer (poorly differentiated adenocarcinoma with diffuse cytoplasmic staining against anti-VEGF-C antibody).

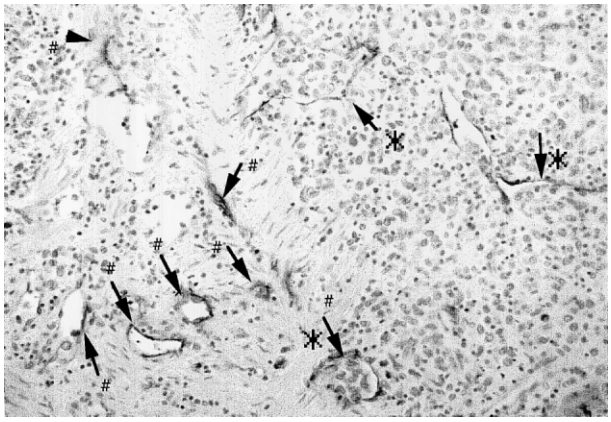

Fig. 4. VEGFR-3-positive lymphatic vessels in the stroma of a primary tumour (#). Cancer cell in the lumen of lymphatic vessel (*).

Next, the number of VEGFR-3-positive vessels in the stroma of the primary tumour were counted under a microscopic field of ×400 magnification. The number of VEGFR-3-positive vessels in the normal gastric mucosa was 2.48±1.64, and was fewer than that in the tumour stroma (4.62±5.85) ($P=0.067$).

The number of VEGFR-3-positive vessels in the *VEGF-C* mRNA-positive tumours (6.96 ±6.05) was significantly greater than that of *VEGF-C* mRNA-negative tumours (2.16±2.00) (Table 3). Furthermore,

Table 3
Correlation between pathological factors and the number of VEGFR-3-positive vessels in primary tumours

Pathological factors	No. of cases	No. of VEGFR-3-positive vessels	
VEGF-C mRNA (RT-PCR)			
Not detected	38	2.16 ±2.00	$P<0.001$
Detected	47	6.96 ±6.05	
Histological type			
Differential type	43	3.66 ±3.63	$P=0.045$
Poorly differentiated type	42	6.67 ±6.55	
Lymph node metastasis			
Negative	27	2.91 ±1.99	$P=0.011$
Positive	58	5.67 ±5.66	
Lymphatic invasion			
Negative	37	3.27 ±3.23	$P=0.013$
Positive	48	5.93 ±5.91	
Venous invasion			
Negative	36	4.67 ±6.67	NS
Postive	49	5.11 ±4.35	
Serosal invasion			
Negative	42	4.71 ±4.24	NS
Positive	43	5.18 ±6.13	

RT-PCR, reverse transcriptase polymerase chain reaction; NS, non-significant.

tumours with lymphatic invasion or lymph node metastasis had a significantly greater number of VEGFR-3-positive vessels than did those without involvement in the lymph node or lymphatic vessels. There was a significant difference in the number of VEGFR-3-positive vessels between poorly differentiated adenocarcinoma (6.67 ± 6.55) and differentiated adenocarcinoma (3.66 ± 3.63).

4. Discussion

VEGF-C is known as the lymphangiogenic factor [18] which stimulates mitogenesis in lymphatic endothelial cells through activation of the VEGFR-3 [5]. Recent work of Joukov and colleagues clarified the dual functional activity of VEGF-C not only for lymphangiogenesis, but also in angiogenesis. The mechanism of this phenomenon is now believed to be due to the existence of two receptors for VEGF-C, namely VEGFR-2, and VEGFR-3. The former relates to the angiogenesis and the latter to lymphangiogenesis [20].

The induction of the proliferation of lymphatic vessels by VEGF-C has been confirmed by the experiments using the avian chorioallantoic membrane (CAM) assay [21] and *VEGF-C* transgenic mice [8]. Human recombinant VEGF-C actually induces hyperplasia of lymphatic vessels in the avian chorioallantoic membrane [21]. Jeltsch and colleagues reported that the selective hyperplasia of subcutaneous lymphatic channels was induced in transgenic mice where the *VEGF-C* gene was under the control of the human keratin 14 promoter [9]. Accordingly, the VEGF-C and VEGFR-3 loop may have a pivotal role in lymphangiogenesis.

This activity in proliferating endothelial cells, via the VEGF-C and VEGFR-2 loop, is considered to be active in the embryonic period. Actually, VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of the lymphatic vessels, and the embryonic lymphatic vessels sprout from the endothelial cells of the venous-sac like structure, called the mesonephric vein and anterior cardinal vein, where VEGFR-3 is expressed [5]. In contrast, lymphangiogenesis is repressed in adult tissues, but is activated in special conditions, such as inflammation and cancer [5,19].

In the stroma of human cancer tissues, especially gastric and breast cancers, hyperplasia or dilatation of lymphatic vessels is frequently found. This phenomenon has been considered to be the result of lymphatic obstruction by tumour embolism, but the mechanisms of the lymphangiogenesis in cancer tissues had remained unclear.

The present study clearly demonstrates the activation of the *VEGF-C*/*VEGFR-3* gene in primary gastric cancer. Morphologically, VEGFR-3 expression was exclusively found on the endothelial cells of small vessels in the stroma of primary tumours. The immunohistochemical study also demonstrated that many VEGFR-3-positive vessels with small diameters were found in the stroma of VEGF-C mRNA-positive tumours. Because almost all VEGFR-3-positive vessels were devoid of erythrocytes in their lumen, they were considered as lymphatic vessels.

Furthermore, this study showed a significantly positive correlation between *VEGF-C* mRNA and *VEGFR-3* mRNA expression in primary gastric cancers. Thus, both VEGF-C and VEGFR-3 are expressed in gastric cancer tissues and may be involved in the proliferation of the endothelial cells of lymphatic vessels. Furthermore, the number of lymphatic vessels in VEGF-C-positive tumours was significantly larger than that in VEGF-C-negative tumours. In addition, the number of VEGFR-3-positive lymphatic vessels in the tumour stroma was closely related to the grade of lymphatic invasion.

These results strongly indicate that the VEGF-C produced from the cancer cells may induce the expression of VEGFR-3 on the lymphatic endothelial cells. As a result, lymphangiogenesis must be induced by the activation of the VEGF-C/VEGFR-3 paracrine loop.

In summary, this study shows the close relationship between VEGF-C/VEGFR-3 paracrine loop and lymphangiogenesis in gastric cancer. VEGF-C-producing cancer cells are able to induce lymphangiogenesis in the stroma around them through activation of VEGFR-3 expressed on the endothelial cells of lymphatic vessels. In these circumstances, cancer cells can easily contact with proliferating lymphatic vessels, resulting in the intravasation of cancer cells into the lymphatic vessels, and the establishment of lymph node metastasis.

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