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Original Paper

Cell-retained Isoforms of Vascular Endothelial Growth Factor (VEGF) are Correlated with Poor Prognosis in Osteosarcoma

Y.H. Lee,[1,2] T. Tokunaga,[1] Y. Oshika,[1] R. Suto,[2] K. Yanagisawa,[2] M. Tomisawa,[1] H. Fukuda,[2] H. Nakano,[3] S. Abe,[3] A. Tateishi,[3] H. Kijima,[1] H. Yamazaki,[1] N. Tamaoki,[1] Y. Ueyama[1] and M. Nakamura[1]

[1]Department of Pathology; [2]Department of Orthopaedics, Tokai University School of Medicine, Bohseidai, Isehara-shi, Kanagawa 259-1193; and [3]Department of Orthopaedic Surgery, Teikyo University School of Medicine, Tokyo, Japan

Vascular endothelial growth factor (VEGF) is a major angiogenic factor. Osteosarcoma is characterised by hypervascularity and metastatic potential. We examined VEGF mRNA expression, VEGF isoform pattern and VEGF receptor (flt-1 and KDR) by RT-PCR analysis in 30 osteosarcomas. All 30 osteosarcomas expressed VEGF mRNA. 17 osteosarcomas (57%) expressed flt-1 mRNA, whilst 20 (67%) expressed KDR mRNA. 6/30 (20%) osteosarcomas were positive for VEGF121 only, 8 (27%) for VEGF121 + VEGF165, and 16 (53%) for VEGF121 + VEGF165 + VEGF189. Patients with osteosarcomas with VEGF165 ($n = 24$) had significantly poorer prognosis in comparison with those without VEGF165 ($P = 0.022$, Wilcoxon's test). The osteosarcomas with VEGF165 had significantly increased vascularity assessed on sections immunostained for CD34 ($P < 0.001$, Mann–Whitney U test). Although VEGF165 is a soluble isoform, it is also retained on the cellular surface. These results suggest that cell-retained VEGF isoforms (VEGF165, VEGF189) might be essential for neovascularisation in osteosarcoma, whilst the soluble VEGF121 isoform is not sufficient to stimulate neovascularisation in this type of neoplasm. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

OSTEOSARCOMA CAUSES high morbidity in young adults and adolescents. Despite intensive treatment, including adjuvant chemotherapy, wide excision of tumours and amputation of the affected limbs, approximately half such patients die within 5 years [1]. High metastatic potential and high recurrence rate are considered to be associated with high levels of vascularisation and rapid growth of these tumours [2].

Growth, progression and metastasis of solid tumours are dependent on neovascularisation [3]. Vascular endothelial growth factor (VEGF) is a major angiogenic factor that induces endothelial cell proliferation and increases the permeability of the vascular endothelium [4, 5]. Targeted disruption of the VEGF gene has indicated that VEGF is indispensable to new blood vessel formation and growth [6]. VEGF is generally considered to play an important role in neovascularisation of tumours. VEGF expression has been shown to be correlated with vascular density in invasive ductal carcinoma and human epidermoid lung carcinoma [7, 8]. Enhanced VEGF gene expression has been reported in a number of malignant tumour cell lines as well as in primary tumours from breast, lung, ovarian, liver and colon cancer in comparison with normal tissue [7–12].

Five isoforms of VEGF (VEGF121, VEGF145, VEGF165, VEGF189, VEGF206) are generated by alternative splicing [13]. VEGF121, VEGF165 and VEGF189 expression have been reported in some types of tumours. However, there have been few studies of VEGF145 expression [14, 15]. It is also unclear whether VEGF206 is expressed in adult tissue. These VEGF isoforms seem to possess different biological properties [16, 17]. VEGF121 proteins is secreted in soluble form, while VEGF145, 189 and 206 are bound to cell-surface heparin-containing proteoglycans in

Correspondence to M. Nakamura; e-mail: mnakamura@is.icc.u-tokai.ac.jp
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the extracellular matrix [14, 18]. VEGF165 has been shown to be expressed in both forms *in vitro* [19]. We have reported previously that VEGF isoform patterns differ with tumour type, while expression patterns of VEGF isoforms are closely associated with progression of carcinomas including colon cancer, non-small cell lung cancer (NSCLC) and renal cell cancer [12, 20–23]. There are marked embryological differences between epithelial malignant neoplasms (carcinoma) and non-epithelial malignant neoplasms (sarcoma). It is unclear to what extent VEGF expression is involved in progression through neovascularisation and which isoforms play major roles in typical non-epithelial malignant neoplasms such as osteosarcoma. In this study, we analysed the correlation between VEGF gene expression, its isoform patterns and their clinicopathological significance in osteosarcoma.

MATERIALS AND METHODS

Specimens

Biopsies from 30 osteosarcomas were obtained (19 in Tokai University Hospital and 11 in Teikyo University Hospital) from 1988 to 1997. None of the patients had received any adjuvant therapy before biopsy. Patients who had distant metastasis at entry were excluded from this study. Median duration of follow-up was 40 months. The biopsies were rapidly frozen and stored at − 80°C until analysis. Total cellular RNA was prepared from frozen specimens.

RT-PCR analysis to detect VEGF isoforms

We evaluated isoforms of VEGF mRNA by RT-PCR according to the previously described procedure [12]. Two alternative primer sets were used as follows: V-S, 5-AAGCCATCCTGTGTGCCCCTGATG-3, and V-S4, 5-CGGATCA AACCTCACCAAGGCC-3, V-A, 5-GCGAATTCCTCCTGCCCGGCTCAC-3, and V-A7, 5-CTTTCTCCGCTCTGAGCAAGGC-3 (Figure 1). Probes (378 bp) were prepared by PCR amplification with primers V-S and V-A, and their sequences were confirmed with an automated sequencer (ABI PRISM 310, Perkin Elmer, California, U.S.A.). Reverse transcription was performed at 42°C for 60 min (1 μg total cellular RNA; 100 pM random primers, Boehringer Mannheim, Germany; reverse transcriptase, GIBCO-BRL, Rockvile, Maryland, U.S.A.). VEGF cDNA fragments were amplified by 30 rounds of PCR consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C with a Gene Amp PCR System 9600 (Perkin Elmer) and Taq DNA polymerase (Toyobo, Osaka, Japan). We also confirmed the existence of PCR fragments derived from exon 6 in isoforms VEGF145, VEGF189 and VEGF206 using a cDNA probe specific for this exon sequence (72 bases). Blots of products (Zeta-Probe, BIO-RAD, Hercules, California, U.S.A.) were hybridised with photochemically labelled probes (ECL; Amersham, Buckinghamshire, U.K.), and exposed to Kodak AR film. The quality of the RNA was estimated by RT-PCR for β2-microglobulin.

Human osteosarcoma xenografts

Nine xenografts were established and maintained by serial subcutaneous transplantation in SCID mice (Clea Japan Inc., Tokyo, Japan, 10–20 passages). We obtained xenografts from mice sacrificed under deep anaesthesia. No significant morphological differences were observed between xenografts and primary tumours.

RT-PCR analysis to detect VEGF receptors flt-1 and KDR

VEGF receptor gene expression (flt-1 and KDR) was estimated by RT-PCR under conditions similar to those described above according to the previously described procedure [12] using the following primers: flt-1 sense, 5-ATGAGCAGTGTGAGCGGCTCCC-3 (2669-2690); flt-1 antisense; 5-AAGCTTTCGCTGCTGGTGACGC-3 (3125-3146); KDR sense, 5-CGTCATGGATCCAGATGAACTCCC-3 (2406-2429); KDR antisense, 5-CTTGACGGAATCGTGCCCCTTTGG-3 (2813-2836).

Vascularisation in osteosarcoma

Estimation of vascularity was performed as described previously [23]. Formalin-fixed (10%), paraffin-embedded sections of the tumour tissue were immunohistochemically examined with murine antihuman CD34 monoclonal antibody (NCL-end, Novo Castra, U.K.). After blockage of endogenous peroxidase activity (methylalcohol, 3% H_2O_2) and nonspecific binding (10% normal goat serum), specimens were incubated with the antibody (1:20) at room temperature for 60 min. Sections were serially incubated with biotin-labelled anti-murine IgG and horseradish peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan). Reaction products were visualised with 3,3′-diaminobenzidine. Light microscopy was used to identify 3 regions within or immediately adjacent to the tumour containing the highest numbers of vessels [24]. The vessel counts were evaluated at ×200 magnification (×20 objective and ×10 ocular, 0.739 mm^2 per field) using a computerised image analysis system (Interactive Build Analysis System, Zeiss, Germany).

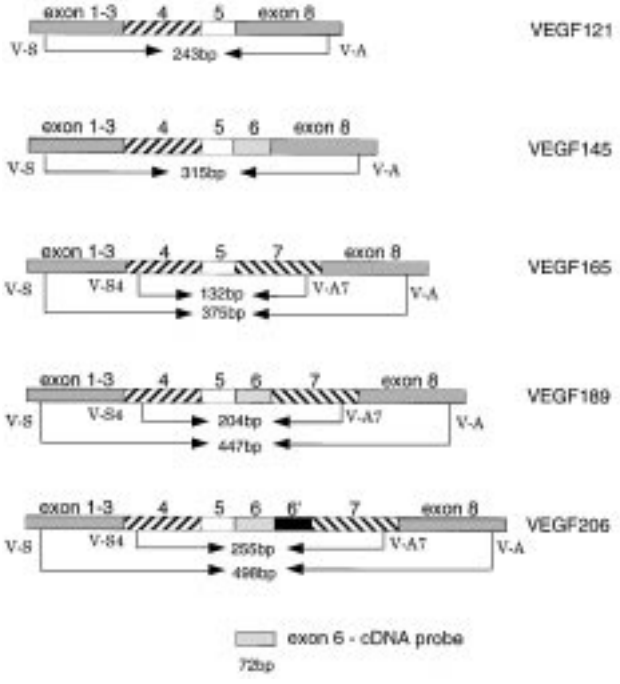

Figure 1. Primers for detection of the isoforms of VEGF mRNA: arrows indicated the sites of primers V-S, V-A, V-S4 and V-A7. PCR with V-S and V-A yielded VEGF121 (243 bp), VEGF145 (315 bp), VEGF165 (375 bp), VEGF189 (447 bp) and VEGF206 (498 bp) fragments. PCR with V-S4 and V-A7 yielded VEGF165, VEGF189 and VEGF 206 fragments. Confirmation of PCR fragments from exon 6 in isoforms VEGF145, VEGF189 and VEGF206 using cDNA probe specific for the exon 6 sequence (72 bp).

Statistical analysis

Fisher's exact test or χ^2 test was applied for comparisons between group frequencies. Differences in survival between subgroups of patients were compared with the Wilcoxon's test, and survival curves were plotted according to the method of Kaplan and Meier. The statistical significance of differences in mean vessel counts between the groups were examined by Mann–Whitney U test.

RESULTS

Expression of VEGF, its isoform patterns and VEGF receptors (flt-1, KDR)

VEGF mRNA was detected in all 30 patients with osteosarcoma. Six of the 30 osteosarcomas expressed VEGF121 only (20%). Eight of the 30 specimens expressed VEGF121 + VEGF165 (27%), and 16 specimens expressed VEGF121 + VEGF165 + VEGF189 (53%) (Figure 2). All 9 osteosarcoma xenografts were positive for VEGF gene expression, with the isoform expression pattern VEGF121 + VEGF165 + VEGF189. VEGF145 and VEGF206 were not detected in any specimen. 17/30 (57%) specimens expressed flt-1 mRNA and 20/30 (67%) specimens expressed KDR mRNA. There was no significant correlation between VEGF isoform pattern and VEGF receptor expression.

Correlation between VEGF mRNA isoform expression pattern and clinical characteristics

All patients underwent surgery within 3 months of their biopsy. The specimens obtained by surgery were histologically examined by two pathologists according to the pTNM criteria [25] (Table 1). There was no association between VEGF165 expression and histological subtype/staging (Table 1). Overall, metastases was seen in 21/30 (70%) patients during the follow-up period. 19 of these 21 patients died due to pulmonary metastases. There was a significant correlation between the presence of metastases and VEGF165 expression ($P = 0.005$, Fisher's exact test, Table 1). The patients with osteosarcoma expressing the VEGF165 isoform ($n = 24$) showed significantly poorer prognosis than those without this isoform ($P = 0.022$, Wilcoxon's test, Figure 3). There were no correlations between expression of VEGF receptors (flt-1, KDR) and any clinical feature.

Table 1. Patient characteristics and univariate analysis of the associations between VEGF isoform pattern and patient or tumour characteristics

	VEGF165 +	VEGF165 –	*P*-value
Histology			>0.05
Osteoblastic	10	2	
Fibroblastic	4	3	
Chondroblastic	6	0	
Mixed	4	0	
Other	0	1	
Stage (pTNM)			0.20
IB	0	1	
IIB	24	5	
Vessel counts	37.1±19.3	3.9±2.8	<0.001*
Recurrence			1.0
Yes	5	1	
No	19	5	
Metastasis			0.005†
Yes	20	1	
No	4	5	
flt-1			1.0
Yes	14	3	
No	10	3	
KDR			0.37
Yes	17	3	
No	7	3	

*VEGF165 expression was significantly correlated with vessel counts and †metastasis in osteosarcoma ($P<0.001$, Mann–Whitney U test, $P = 0.005$; Fisher's exact test, respectively).

Vascularisation and VEGF isoform expression

The mean vessel count was 29.6 ± 22.0 per ×200 fields (range 1–83). The mean vessel count in tumours without VEGF165 was 3.9 ± 2.8 (range 1–12), whilst for those positive for VEGF165 it was 37.1 ± 19.3 (range 4–83). The difference between these groups in vessel count was significant ($P<0.001$, Mann–Whitney U test, Table 1, Figure 4). However, there were no correlations between VEGF receptor expression (flt-1, KDR) and vascularisation.

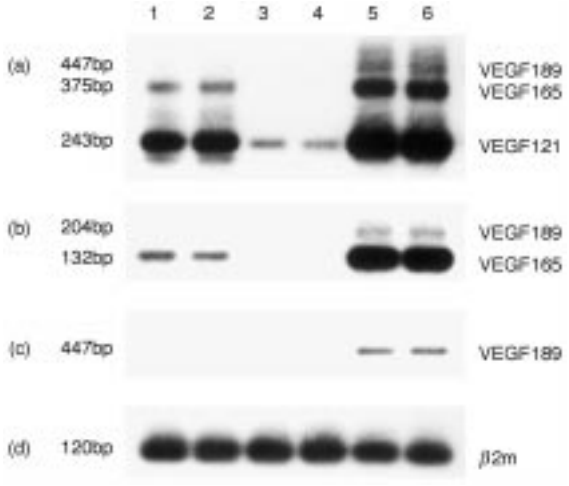

Figure 2. VEGF mRNA isoform expression pattern determined by RT-PCR using V-S, V-A (a), V-S4, V-A7 (b), Exon 6 cDNA probe (c): Lanes 1, 2; VEGF121+VEGF165, Lanes 3, 4; VEGF121, Lanes 5, 6; VEGF121+VEGF165+VEGF189. (d) β2 Microglobulin (β2m) gene expression was evaluated as a control for RNA quality.

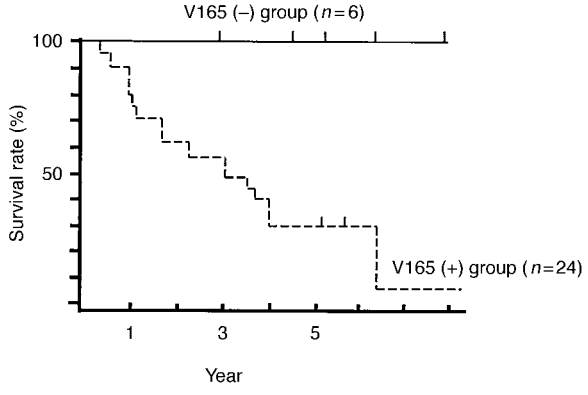

Figure 3. Overall survival according to VEGF165 mRNA expression. Patients with tumours expressing VEGF165 had a poorer prognosis than those without VEGF165 expression (generalised Wilcoxon's test, $P = 0.022$).

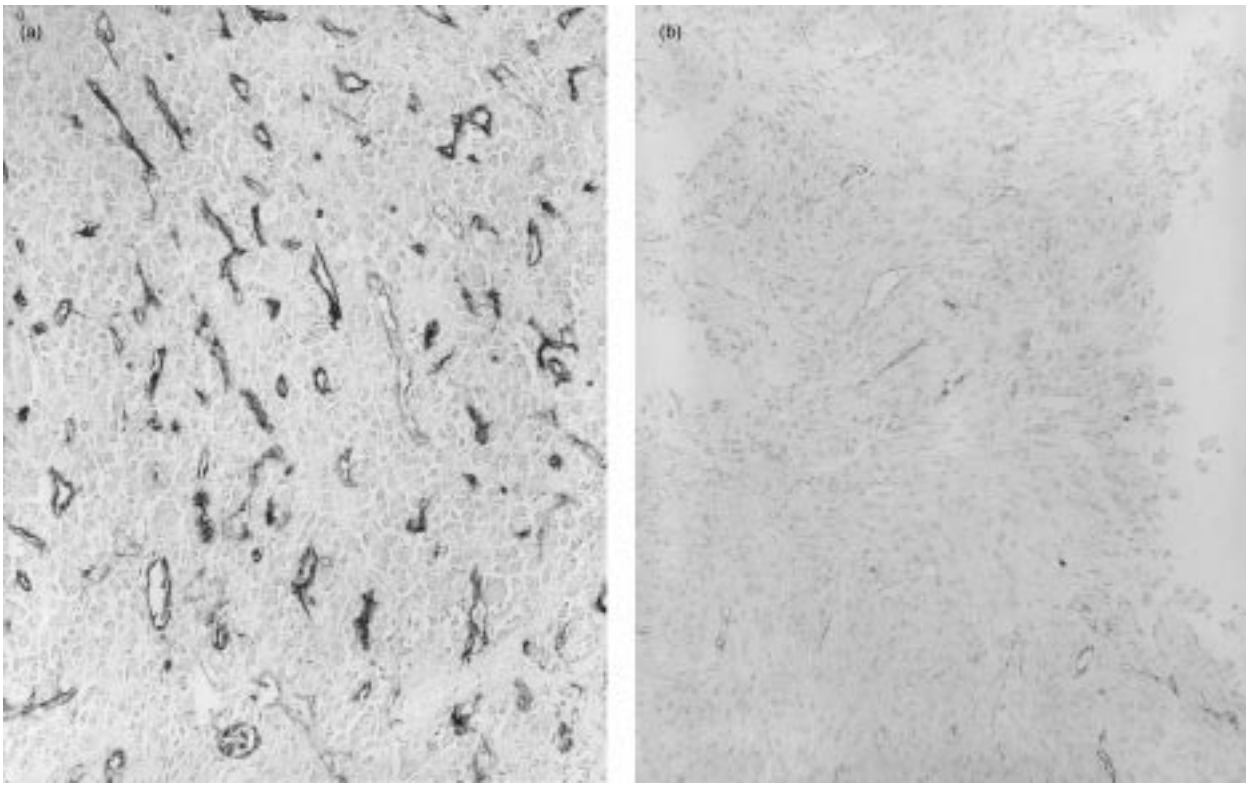

Figure 4. Vascularisation in an osteosarcoma was demonstrated by immunostaining for CD34. (a) Specimens with VEGF165 showed significantly increased vessel counts. (b) Specimens without VEGF165 showed sparse vascular tissue. (×150).

DISCUSSION

Osteosarcoma with prominent vascularisation may lead to distant metastasis. Tumour growth, progression and metastasis are dependent on new blood vessel formation (neovascularisation). VEGF is a potent angiogenic factor secreted by a variety of tumour cells [8–12, 21]. There have been no reports concerning VEGF expression in primary samples from osteosarcoma, whilst a few studies have mentioned VEGF gene expression level in osteosarcoma cell lines [26, 27]. We detected VEGF transcripts in all primary osteosarcoma and xenograft specimens examined, indicating that VEGF mRNA is expressed in osteosarcoma cells.

VEGF145, 165, 189 and 206 possess strong affinity to cell-surface heparin-containing proteoglycans, whilst VEGF121 protein does not [14, 17, 18]. However, both VEGF121 and 165 protein have been detected in culture medium as secretory proteins [16, 19]. Each isoform of VEGF including VEGF165 plays a role in vascular permeability. All the osteosarcoma specimens expressed VEGF121, 24 of the 30 specimens (80%) showed VEGF165 expression, and 16 (53%) showed VEGF189 expression in this study. Neither VEGF145 nor VEGF206 was detected in osteosarcoma. VEGF189 expression was found at a significantly lower incidence in our osteosarcoma specimens (16/30) than in NSCLC (76/84) ($P<0.01$, χ^2-test) in our previous study [21].

The prognostic significance of neovascularisation in solid neoplasms has been demonstrated in several kinds of tumour including malignant melanoma, breast, bladder and NSCLC [24, 28, 29]. In this study, we demonstrated significant correlations between VEGF165/VEGF165 + VEGF189 expression and increased vascularisation and poor prognosis in osteosarcoma. VEGF121 and VEGF165 are thought be the most abundant isoforms of VEGF. The cellular-retained isoforms VEGF165 and VEGF189 differ from VEGF121 because of an ability to bind cell-surface proteoglycan. These results suggest that VEGF121 alone, which is released from cells, may be insufficient, whilst cell-retained VEGF isoforms (VEGF165, VEGF189) are essential for neovascularisation of osteosarcoma.

VEGF is considered to be an endothelial cell-specific growth factor. However, there have been some reports of interactions of VEGF with non-endothelial cells via isoform-specific receptors other than flt-1 or KDR [30]. It has been reported that migration of osteoblasts is stimulated by VEGF [31]. In the present study, we found no significant correlation between isoform pattern of VEGF and VEGF receptor (flt-1, KDR) expression. The hypothesis that this migration is mediated by a VEGF165 isoform-specific receptor on the cell surface of osteoblasts is compatible with our observation that VEGF165 expression was correlated with poorer prognosis of osteosarcoma patients. These results are in contrast to those of our previous studies indicating that the VEGF189 isoform is correlated with malignant progression in some types of epithelial malignant neoplasms including colon, NSCLC and renal cell carcinoma. This is probably because osteosarcomas originating from non-epithelial tissue elements are different from tumours of epithelial origin with regard to the contribution of VEGF isoforms to growth of the neoplasms. Cell-retained VEGF isoforms (VEGF165, VEGF189) may act not only as angiogenic factors but also as growth stimulatory factors via autocrine and/or paracrine mechanisms using tumour cell-surface receptors in osteosarcomas.

In conclusion, the results presented here suggested that cell-retained VEGF isoforms (VEGF165, VEGF189) may be

essential for the growth of osteosarcoma. Although the present study was small and the results presented here are preliminary observations, examination of the VEGF mRNA isoform patterns will be helpful for predicting the prognosis of patients with osteosarcoma. Further analysis of VEGF isoform-specific receptors is required to understand the possible paracrine or autocrine mechanisms of action of VEGF in osteosarcoma.

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