

Expression of VEGF and its Receptors and Angiogenesis in Bone and Soft Tissue Tumors

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Abstract. *Background:* Tumor angiogenesis and vascularization are essential requirements for the growth and metastasis of tumors. There is evidence that overexpression of the vascular endothelial growth factor (VEGF) is correlated with an adverse prognosis in some tumors. The expression of VEGF, its receptors and microvessel density (MVD) of bone and soft tissue tumors was evaluated. *Materials and Methods:* Tissue specimens of 60 patients including 30 malignant and 30 benign tumors confirmed by biopsy were examined. Expression of VEGF and its receptors (flt-1 and KDR/flk-1) was observed by immunohistochemistry. Tumor angiogenesis was assessed morphologically by measuring intratumoral MVD. *Results:* Semi-quantitative evaluation of immunoreactivity showed that VEGF was significantly higher in malignant tumors than in benign tumors. A correlation was found between the immunoreactivity of VEGF and KDR. Moreover, correlations were found either between MVD and VEGF or between MVD and KDR/flk-1. *Conclusion:* Signal transduction, in particular by VEGF and KDR, potentially contributes to the angiogenesis of bone and soft tissue tumor.

Angiogenesis within the tumor is an indispensable process for the growth and metastasis of neoplasms. Of the multitude of growth factors that regulate physiological and pathological angiogenesis, vascular endothelial growth factor (VEGF) is believed to be the most important (1), but its role as a predictor of metastasis and survival is yet to be defined. VEGF and its receptors (flt-1 and KDR/flk-1) have been

demonstrated to be good markers of angiogenesis. There is evidence that overexpression of VEGF is correlated with an adverse prognosis, at least in some tumors, such as colon and rectal cancers (2), liver cancer (3), lung, thyroid, breast, gastrointestinal tract, kidney and bladder cancers, ovary and uterine cervix carcinomas, angiosarcomas, germ-cell tumors and intracranial tumors (4). A variety of agents aimed at blocking VEGF or its receptor-signaling system are currently being developed for the treatment of cancer (1). Tumor angiogenesis is assessed morphologically by measuring intratumoral microvessel density (MVD). VEGF expression has been found to correlate with MVD. The association between VEGF and its receptors and tumor grade and MVD has been reported (5, 6). However, there are only a few reports concerning bone and soft tissue tumors. The aim of the present study was to evaluate the association between VEGF and its receptor system and the MVD of bone and soft tissue tumors, both malignant and benign.

Materials and Methods

Specimens. Tissue samples were obtained from 60 bone and soft tissue tumors (30 malignant tumors and 30 benign tumors; Table I) by biopsy from 2001 to 2006 at Kobe University Hospital. All the human specimens used were obtained with informed consent. None of the patients received any adjuvant therapy before biopsy. The biopsy specimens were rapidly frozen and stored at -80°C until analysis.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections by the indirect immuno-peroxidase method for flt-1, KDR/flk-1, VEGF and CD34. The tumor sections were immunostained with human flt-1 polyclonal antibody (Neo Markers, Lab Vision Corporation, California, USA), KDR/flk-1 monoclonal antibody (Santa Cruz Biotechnology, Inc., California, USA), VEGF polyclonal antibody (Neo Markers, Lab Vision Corporation) and CD34 monoclonal antibody (Nichirei Biosciences, Tokyo, Japan). The sections were deparaffinized with xylene and routinely dehydrated through a series of graded alcohols.

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Table I. Tissue samples obtained from 60 bone and soft tissue tumors.

Malignant tumors		Benign tumors	
Liposarcoma	10	Shwannoma	12
Osteosarcoma	7	GCT	8
MFH	6	Lipoma	5
Ewing's sarcoma	2	Enchondromatosis	1
Chondrosarcoma	2	Solitary fibrous tumor	1
Synovial sarcoma	2	Neurofibroma	1
Leiomyosarcoma	1	Langerhans cell histiocytosis	1
		Chordoma	1
Total	30		30

MFH= malignant fibrous histiocytoma;

GCT= giant cell tumor.

For flt-1, KDR/flk-1 and VEGF immunostaining, antigen unmasking in the sections was performed by pre-treatment using Proteinase K for 10 min. For CD34 immunostaining, the unmasking was performed by heat treatment for 20 min. Following elimination of endogenous peroxidase activity with a 10-min incubation in 3% H₂O₂, the sections were incubated at room temperature for 1 h with primary antibodies against flt-1 (1:200) and KDR/flk-1 (1:100), or at room temperature overnight with primary antibodies against VEGF (1:200) and CD34 (100 µl/one slide). After washing with Tris-buffered saline (TBS), the sections were then incubated at room temperature for 30 min with goat anti-rabbit immunoglobulins for flt-1 and VEGF, or anti-mouse immunoglobulins for KDR/flk-1 and CD34, conjugated to peroxidase-labeled amino acid polymer (Nichirei Biosciences). After washing with TBS, 3,3'-diaminobenzidine tetrahydrochloride was used for color development and the sections were counterstained with hematoxylin. Negative controls included incubations with irrelevant class-matched and species-matched immunoglobulins and incubations in which the primary antibody were omitted.

Evaluation of immunohistochemistry. A semi-quantitative system was employed to evaluate the level of antigen expression: Immunoreactivity was scored as either negative (0), focal (1+, less than 25% of positive cells), moderate (2+, 25-50% of positive cells), or diffuse (3+, more than 50% of positive cells). The intensity of immunostaining was rated as follows: none (0), weak (+1), moderate (+2) and intense (+3). The IHC score was defined as the sum of the two scores above. The specimens were evaluated by two observers (HH, TA) and finally scored by consensus of the observers.

Determination of MVD. At low power field (x100), the tumorous tissue sections were screened and the three areas with the most intense neovascularization (hot spots) were selected. Microvessel counts of these areas were performed at high power field (x400). Any CD34 positive endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells and connective tissue elements was considered single countable microvessel; branching structures were counted as one, unless there was a break in the continuity of the vessel, in which case it was counted as two distinct vessels. Three fields per tumor section were counted in the areas that appeared to contain the greatest number of microvessels on scanning at low magnification. Microvessel density was defined as the mean score from all three fields.

Statistical analysis. The statistical significance of the individual findings and their association indices were evaluated by the Mann-Whitney *U*-test, the Chi-square test or Spearman's rank-order correlation coefficient. Probability, *p*-values less than 0.05 were considered significant.

Results

Immunostaining. Positive immunostaining of VEGF, VEGF receptors (flt-1, KDR/flk-1) and CD34 are shown in Figure 1. A semi-quantitative evaluation of immunoreactivity results showed that VEGF was significantly higher in malignant than benign tumors (*p*=0.006) (Figure 2), whereas flt-1 and KDR/flk-1 were not significantly different. Moreover, a correlation was found between the immunoreactivity of VEGF and KDR/flk-1 (*r*=0.369, *p*=0.004) (Figure 3). The mean (±S.D.) MVD for all tumors was 19.3 (±16.2). The mean (±S.D.) MVD for malignant tumors was 27.6 (±15.6) and 11.1 (±12.3) for benign tumors. The difference was statistically significant (*p*<0.0001). In addition, correlations were found either between MVD and the immunoreactivity of VEGF (*r*=0.513, *p*<0.0001) or KDR/flk-1 (*r*=0.415, *p*=0.001), whereas no correlation was found between MVD and the immunoreactivity of flt-1 (Figure 4).

Discussion

The angiogenesis of tumors is a highly regulated process influenced by the host microenvironment and mediators. Neovascularization in solid tumors correlates with metastasis and recurrence. VEGF and its receptors, flt-1 and KDR/flk-1, were identified as providing an important regulating system for tumor angiogenesis by performing a paracrine-autocrine role (1). VEGF is a dimeric glycoprotein, that acts as a mitogen for endothelial cells and induces angiogenesis and changes in vascular permeability and is involved both in tumor vascularization and growth (8-10). VEGF can have an impact on survival by inhibiting apoptosis (11, 12). VEGF exerts its effects by binding with high affinity to two tyrosine kinase receptors, which are expressed on the surface of endothelial cells; flt-1 and KDR/flk-1 (8-10, 13-17). KDR/flk-1 is responsible for the proliferative response of endothelial cells to VEGF, whereas flt-1 is responsible for vascular tabulation and maturation (18, 19). In the VEGF-VEGF receptor system, flt-1 is only one-tenth of KDR/flk-1 when its activity and autophosphorylation of tyrosine kinase is compared, although flt-1 is more than 10 times stronger than KDR/flk-1 when its strength is combined with VEGF-A. This suggests that KDR/flk-1 carries the main signal transduction and that flt-1 serves a regulatory role. KDR/flk-1 directly contributes to formations, such as tumor angiogenesis, while flt-1 contributes to tumor metastasis and inflammatory

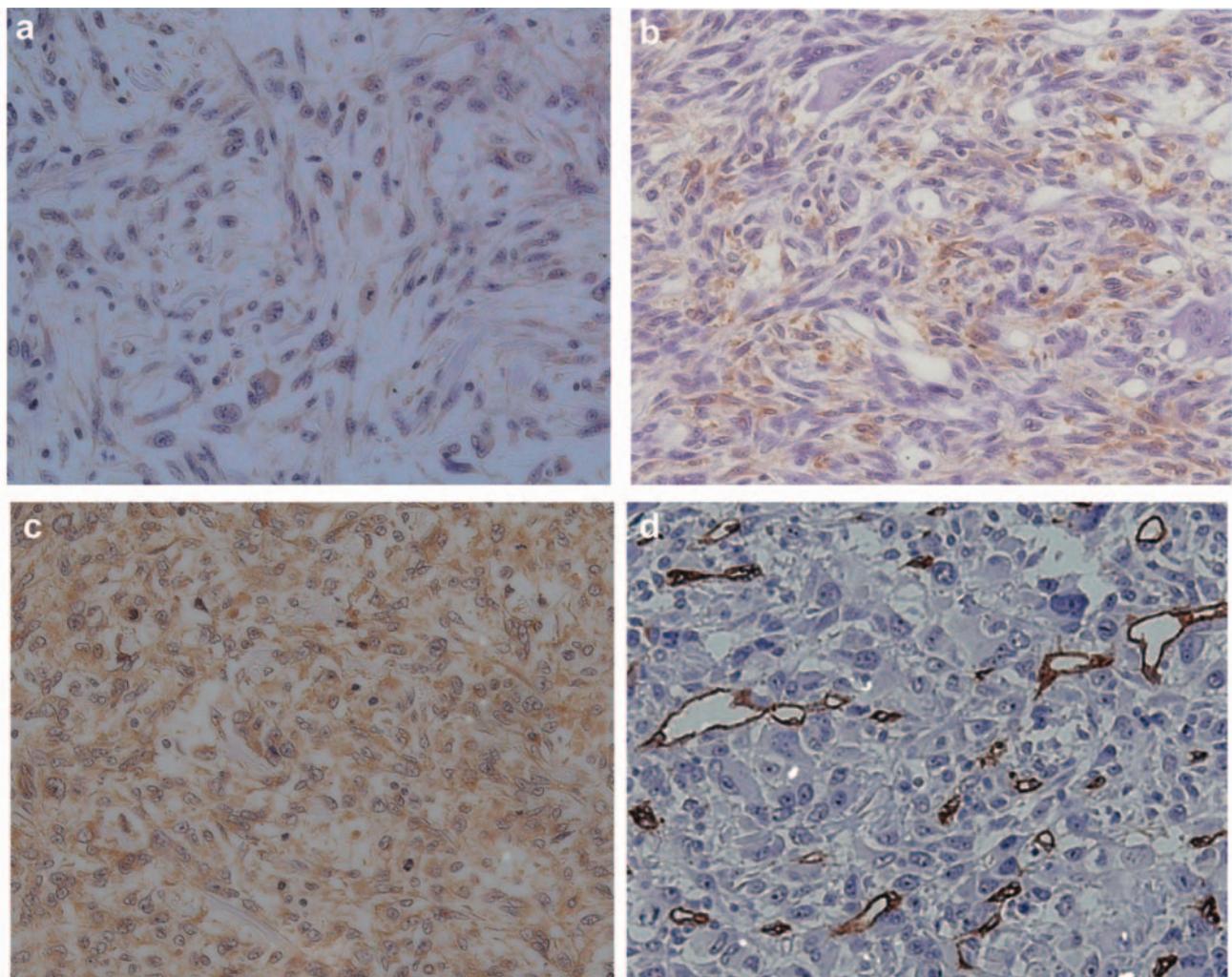


Figure 1. Positive immunohistochemical detection (x400). Positive immunostaining of VEGF receptors: flt-1(a), KDR/flk-1(b), VEGF (c) and CD34 (d) are shown.

disease through the macrophage system (20). Expression of VEGF receptors was reported in a variety of non-endothelial cells, including cancer cells (21-25). A number of studies have found an increase in flt-1 and KDR/flk-1 expression induced by exogenous VEGF in human vascular endothelial cells (26, 27), other endothelial cells (28) and even in tumor cells (29). Beierle *et al.* reported that the difference in VEGF receptor expression was demonstrated at both mRNA and protein levels and they suggested that the biological effects of VEGF upon the tumor may depend on the types of receptor present, and, further, that those growth factors and environmental conditions may alter the level of expression of these receptors (30). In our study, VEGF was significantly higher in the malignant than the benign tumors and there was a correlation between VEGF and KDR/flk-1 by immunohistochemistry evaluation. In several reports, the angiogenesis of tumors was assessed

morphologically by measuring the intratumoral MVD with various endothelial markers, such as CD34, CD31 and factor VIII. However, the relationship between MVD and VEGF in soft tissue sarcomas has not been extensively examined and the results of a small number of reported studies are still controversial (31-33). In contrast, in this study significantly higher MVD in malignant than benign tumors and additional correlations either between MVD and VEGF expression or MVD and KDR/flk-1 expression were found, although no correlation was found between MVD and flt-1 expression. Based on these results, we suggest that signal transduction, in particular by VEGF and KDR/flk-1, contributes to the angiogenesis of bone and soft tissue tumors. Although the high expression of VEGF in tumor cells correlated with poor outcomes in prostate carcinoma, breast carcinoma and lung carcinoma (34), the role of VEGF in soft tissue sarcomas was examined in a few

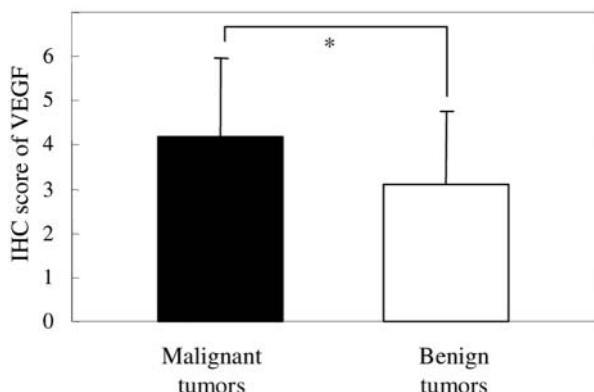


Figure 2. Results of semi-quantitative evaluation of immunoreactivity. The mean (\pm S.D.) IHC scores for malignant and benign tumors are 4.17 (\pm 1.80) and 3.10 (\pm 1.67). *VEGF is significantly higher in malignant than benign tumors ($p=0.006$).

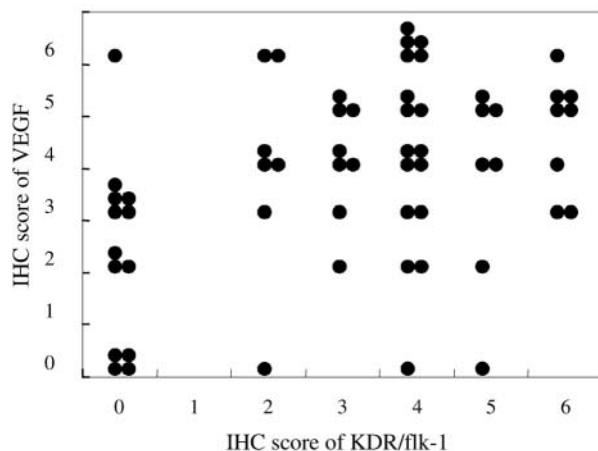


Figure 3. Correlation of VEGF and KDR/flk-1 expression.

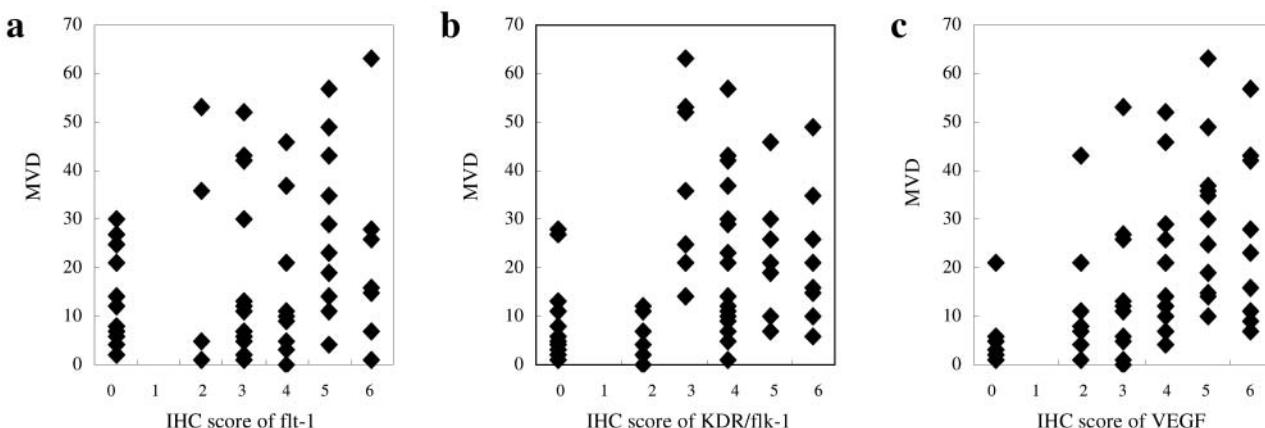


Figure 4. Correlation between MVD and expression of VEGF and VEGFRs.

studies with controversial results (7, 8, 31, 35). However, conclusions cannot be drawn from the published literature, because a remarkable heterogeneity seems to exist among the studies regarding the histological subtypes of soft tissue sarcomas, including the chosen cut-off levels of VEGF positivity, as well as the methodology of VEGF evaluation (6). The correlation between VEGF expression and clinical prognosis cannot be tested, due to a short-term of follow up for patients. Further evidence of a relationship between VEGF-KDR/flk-1 and the degree of malignancy in bone and soft tissue tumors should be provided. However, we also raise the possibility that VEGF-KDR/flk-1 is an angiogenesis-related factor in bone and soft tissue tumors. Further research using a large, prospective and homogenous group of patients is needed to fully determine the relationship between angiogenesis and clinical outcomes.

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