Vascular Endothelial Growth Factor Expression in Untreated Osteosarcoma Is Predictive of Pulmonary Metastasis and Poor Prognosis

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ABSTRACT

To investigate the clinical significance of vascular endothelial growth factor (VEGF) in osteosarcoma, we immunohistochemically stained biopsy specimens of 27 primary osteosarcomas using an antibody against VEGF and evaluated the correlation between the expression of VEGF and local density of CD34-positive microvessels, clinicopathological variables, and survival of patients. VEGF staining was positive in 17 tumors (63.0%) in which the density of CD34-positive microvessels was significantly higher than that in VEGF-negative 10 tumors ($P < 0.05$). In terms of clinicopathological variables, there was no correlation between the expression of VEGF and histological subtype, stage, or response to neoadjuvant chemotherapy, or, strikingly, to the development of pulmonary metastasis (89% of VEGF-positive tumors versus 10% of VEGF-negative tumors; $P < 0.0003$). Moreover, patients with a VEGF-positive tumor were poorer in both disease-free survival ($P < 0.001$) and overall survival ($P < 0.03$) compared to those with a VEGF-negative tumor. These findings strongly suggest that VEGF expression in untreated osteosarcoma is predictive of pulmonary metastasis and poor prognosis in patients who underwent aggressive therapy and also provide the basis for a therapeutic strategy targeting angiogenic property of osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common malignant bone tumor in adolescents and young adults. Despite recent advances in multimodality treatments consisting of aggressive adjuvant chemotherapy and wide tumor excision, pulmonary metastasis occurs in approximately 40–50% of patients with osteosarcoma and remains a major cause of fatal outcome (1).

Tumor metastasis is a multistep process involving a variety of tumor cell-host cell interactions. Angiogenesis, one such interaction, elicits proliferation and migration of endothelial cells to allow the formation of new capillaries (2). Because solid tumors require neovascularization for continuous growth both at the primary and the metastatic sites, the angiogenic phenotype is of fundamental importance for tumor cells to establish metastasis. It has become increasingly clear that solid tumors produce several growth factors that have angiogenic properties, thereby exerting their angiogenic phenotype in the microenvironment (2, 3).

VEGF is a homodimeric protein recently identified as a mitogen for endothelial cells in vitro and an angiogenesis-promoting factor in vivo (4, 5). VEGF activates endothelial cells via interaction with its receptors, Flk-1 and Flt-1, which are selectively expressed in the endothelium (6, 7). The importance of VEGF in tumor metastasis has been indicated by the correlation between VEGF expression in the primary tumor and the metastatic rates, as well as the poor prognosis for patients with certain malignant tumors, such as gastric carcinoma (8), colorectal carcinoma (9), or esophageal carcinoma (10). With regard to osteosarcoma, there is a study using a murine model showing the overexpression of VEGF mRNA in a highly metastatic osteosarcoma cell line (11). However, the clinical relevance of this animal study has not yet been demonstrated.

In this article, we directed our attention to the clinical significance of VEGF expression and addressed the association of VEGF expression in the primary tumor with local microvesSEL density, clinicopathological features, and the prognosis for patients with osteosarcoma.

MATERIALS AND METHODS

Clinical Materials. This study was approved under our institutional guidelines for the use of human subjects in research. Eligible patients for this analysis had a newly diagnosed primary osteosarcoma, had not received irradiation or chemotherapy before biopsy, and completed treatment protocols consisting of wide or radical tumor excision with multiagent adjuvant chemotherapy or a combination of adjuvant and neo-
adjuvant chemotherapy. Paraffin-embedded biopsy specimens from 27 patients who met the above-mentioned criteria were analyzed. The patients ranged in age from 9 to 82 years (average age, 24.9 years). Fourteen were men, and 13 were women. Fifteen of the tumors were located in the femur, 4 in the tibia, 3 in the humerus, 2 in the fibula, and 1 each in the forearm, hand, and pelvis. At final follow-up, 12 patients died of the disease, 4 patients had no evidence of the disease, and 11 patients were continuously disease free. Nineteen patients received preoperative chemotherapy [Rosen’s T-10 protocol (12), 1 patient; T-12 protocol (1), 10 patients; NSH-7 (1), 8 patients] that was basically the combination of high dose methotrexate, doxorubicin, and cisplatin. The follow-up period for surviving patients was 9.2 years on average (range, 4.3–14.6 years).

After an initial review of all available H&E-stained slides of the biopsy specimens, we selected one representative paraffin block from each case for further study. Consecutive 4-μm sections were recut from each block and immunostained for VEGF and CD34.

**Immunohistochemical Staining.** We carried out immunohistochemical detection of VEGF and CD34 using the avidin-biotin complex method as described previously (13). The primary antibody for VEGF was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution, and the antibody for CD34 was a mouse monoclonal antibody (Nichirei, Tokyo, Japan) at a 1:100 dilution. Normal rabbit or mouse IgG diluted to an equivalent protein concentration served as a control in place of the primary antibody. The polyclonal antibody reactivity for VEGF with individual tissue sections was considered positive if equivalent staining was seen either in

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**Fig. 1 In situ expression of VEGF and CD34 in osteosarcoma tumors.** A, immunostaining for VEGF. In the VEGF-positive case (a and b), more than 80% of tumor cells show positive reaction for anti-VEGF antibody. In the VEGF-negative case (c), no obvious reaction product is seen in tumor cytoplasm. a and c, ×40; b, ×160. B, immunostaining for CD34. In the VEGF-positive case (a), many small vessels positive for CD34 are seen in the stroma of tumor tissue. In the VEGF-negative case (b), CD34-positive vessels were scattered in the tumor stroma that are relatively larger in size and fewer in number than those in the VEGF-positive case. a and b, ×40.
the membrane or the cytoplasm of more than 30% of tumor cells. Immunohistochemical staining for CD34, which was stained in tumor stromal vascular endothelial cells (14), was conducted to evaluate the microvessel density in tumor tissues. The number of CD34-positive vessels was counted in four randomly selected areas of a 1-mm² field, and the average was calculated. As a parameter of total surface area of vasculature, total perimeter of vessels was measured in four randomly selected areas of a 0.25-mm² field using an image analyzer, Carl Zeiss Vision Ibas V. 20 (Zeiss, Germany).

**Evaluation and Statistical Analysis.** The cases were divided into two groups on the basis of positive or negative staining of VEGF, and the number of cases was compared between two groups, as were the microvessel density, clinicopathological variables, and survival of patients. Clinicopathological features included age, sex, histological subtype, stage, response to preoperative chemotherapy, and development of pulmonary metastasis. The biopsy specimens were reviewed by two board-certified pathologists, and histological subtypes was determined according to the criteria of Unni (15). Stage was determined according to the Enneking’s surgical staging system (16). The response to preoperative chemotherapy was evaluated for the surgical specimens of all 19 patients who had received preoperative chemotherapy and divided into good and poor according to the criteria of the Japanese Orthopedic Association (17). Development of pulmonary metastasis was evaluated by a plain X-ray and computed tomography scan taken every 6 months during the follow-up period.

Differences in mean vessel counts were analyzed by Student’s t test. The correlation between clinicopathological variables and the expression of VEGF was statistically analyzed using the χ² test for comparison of two groups. Curves for overall survival and disease-free survival were drawn according to the Kaplan-Meier method, and differences were analyzed by applying the log-rank test. Statistical significance was defined as P < 0.05.

**RESULTS**

**Association of VEGF Expression with Local Microvessel Density.** To investigate the clinical significance of VEGF expression in osteosarcoma, we immunohistologically stained

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**Table 1** Correlation between VEGF expression and clinicopathological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result of immunostaining (no. of patients)</th>
<th>VEGF-negative (n = 10, 37.02%)</th>
<th>VEGF-positive (n = 17, 62.98%)</th>
<th>P</th>
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<tr>
<td>Age</td>
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<tr>
<td>≥ 19</td>
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<td>8</td>
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<tr>
<td>Male</td>
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<td>7</td>
<td>5</td>
<td>&lt;0.05</td>
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<tr>
<td>Female</td>
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<tr>
<td>Histologic type</td>
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<td>Response to chemotherapy</td>
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<tr>
<td>Good</td>
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<td>Pulmonary metastasis</td>
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<td>9</td>
<td>3</td>
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* NS, not significant; NA, not available.
the biopsy specimens of 27 primary osteosarcomas using an antibody specific to VEGF. As representatively shown in Fig. 1, VEGF staining was defined as positive in 17 osteosarcoma tumors (Fig. 1A, a and b) and negative in 10 tumors (Fig. 1A, c). In positive cases, the staining pattern of the tumor was uniform, and VEGF was localized in the cytoplasm and/or membrane of osteosarcoma cells (Fig. 1A, b).

We next assessed the association of VEGF expression and microvessel density in the primary tumor. Immunostaining with the anti-CD34 antibody clearly defined endothelial cells in the microvessels (Fig. 1B, a and b). Notably, the number of vessels counted in VEGF-positive tumors (35.7 ± 9.8) was significantly greater than that in the VEGF-negative tumors (7.2 ± 2.4; Fig. 2A). Furthermore, the vessels that stained for CD34 in the VEGF-positive tumors were smaller in caliber than the vessels that were found in the VEGF-negative tumors (Fig. 1B). Histomorphometric analysis revealed that the total perimeter of vessels was significantly higher in VEGF-positive tumors than in VEGF-negative tumors (Fig. 2B), indicating that the few (but increased in number) vessels induced by VEGF provide a greater surface area of vasculature tumor tissue.

Association of VEGF Expression with Pulmonary Metastasis and Prognosis for Osteosarcoma Patients. We subsequently examined the relationship between VEGF expression and various clinicopathological variables (Table 1). There was no significant correlation between the expression of VEGF in the primary tumor and age, histological subtype, stage of the tumor, or response to preoperative chemotherapy. In contrast, the metastatic rate of the patients with VEGF-positive tumors (14 of 17 patients; 82.35%) was significantly higher than that of those with VEGF-negative tumors (1 of 10 patients; 10.00%; P < 0.0003; Table 1). These results suggested the possibility that VEGF expression might be a metastatic predictive marker of osteosarcoma patients. There was also a significant correlation between the expression of VEGF and sex.

During the follow-up term, 12 patients died of pulmonary metastasis. Fig. 3 shows the disease-free and overall survival rates of patients with positive and negative expression of VEGF. The 5-year survival rate of the patients with negative expression of VEGF was 90.0% for both disease-free survival and overall survival, whereas that of the patients with positive VEGF was 18.5% for disease-free survival and 38.5% for overall survival. The patients with VEGF expression demonstrated poorer survival than the patients without VEGF expression by the log-rank test (P < 0.001 for disease-free survival, P < 0.03 for overall survival).

DISCUSSION

In the present study, we have demonstrated that the expression of VEGF in primary osteosarcoma is correlated with (a) an increase in local microvessel density in the tumor tissue, (b) development of pulmonary metastasis, and (c) poor prognosis for patients with osteosarcoma. These findings suggest that VEGF secreted by osteosarcoma cells elicits angiogenesis, which critically contributes to the development of pulmonary metastasis of osteosarcoma.

Close association between VEGF expression in the primary tumor and poor prognosis has been found in gastric cancer (8), colorectal carcinoma (9), esophageal carcinoma (10), and serous ovarian tumors (18). An increase in the serum level of VEGF was also shown to be a predictive of metastasis in hepatocellular carcinoma (19), gastrointestinal tumors (20), and colorectal carcinoma (20). Given osteosarcoma as an example of mesenchymal cell-derived malignancies, it seems likely that the prognostic significance of VEGF may be a more universal finding than previously presumed, possibly observable in a wide range of malignant tumors, including sarcomas. It should, however, be pointed out that a recent study by Kawauchi et al. (21) demonstrated no correlation between VEGF expression and either microvessel density or prognosis in synovial sarcoma, suggesting that other angiogenesis activators may also play a role in the regulation of angiogenesis in certain sarcomas.

During the process of hematogenous metastasis, VEGF is suggested to play a pivotal role by participating in the regulation of angiogenesis (2, 3). In the primary lesion, VEGF-positive osteosarcoma cells can conceivably facilitate neovascularization through its receptors on the endothelial cells; thus, the tumor
grows more rapidly than a VEGF-negative primary osteosarcoma. It is possible that a greater number of small vessels in the VEGF-positive osteosarcoma tumor enhance the chance of tumor cells shedding into the circulation because newly formed small capillaries that have fragmented basement membrane are more penetrable by tumor cells than mature large vessels (22). In addition, more but smaller vessels provide greater surface area through which the tumor cells can more rapidly metastasize than in tumors that have fewer but larger vessels. At the metastatic sites, osteosarcoma cells with VEGF expression may similarly facilitate neovascularization as at the primary site.

It should be noted that the histological subtype, stage, and response to preoperative chemotherapy were not significantly associated with VEGF expression. The lack of a significant association between VEGF expression and the surgical stage is mainly due to the disproportionate distribution of the cases in the present study, as 23 of the 27 cases (85%) were practically classified into stage IIIB. The response to preoperative chemotherapy has been reported to have a significant prognostic value for osteosarcoma patients (23–26). Because the delivery of the chemotherapeutic reagents to the tumor is largely dependent on blood supply, VEGF-induced neovascularization may in turn have a favorable effect on systemic chemotherapy. Although the reciprocal role of VEGF in tumor growth and chemotherapeutic efficacy remains to be elucidated, the present data support the independent prognostic value of VEGF expression.

With better understanding of the molecular mechanism of angiogenesis, there is growing interest in the use of antiangiogenic agents for treatment of malignant tumors. In experimental animal studies, angiogenesis inhibitors, such as TNP-470 (AGM-1470), successfully suppressed the development of the pulmonary metastasis of osteosarcoma in murine and rat models (27, 28). Now, with the current data from the clinical materials, it is conceivable that the requirement of angiogenesis for tumor dissemination is a common property among osteosarcomas, for which antiangiogenic therapy is likely to be appreciated. Because angiogenesis involves a variety of molecules other than VEGF, further studies are definitely required to determine the appropriate target molecules of this therapeutic strategy.

In conclusion, the present study provided evidence for the prognostic significance of VEGF in osteosarcoma and also the basis for a therapeutic strategy targeting angiogenesis. Because this is a pilot study with a small number of patients, our findings should be further verified in a larger number of cases.

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