The Influence of Dendritic Cell Infiltration and Vascular Endothelial Growth Factor Expression on the Prognosis of Non-Small Cell Lung Cancer

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ABSTRACT
Vascular endothelial growth factor (VEGF) is well known to be produced by many human tumors, and it also plays an important role in tumor neovascularure formation. In addition to angiogenesis promotion, recent basic research has shown that VEGF has another function that allows it to inhibit dendritic cell (DC) maturation. However, very little is known about VEGF-dependent DC inhibition in a clinical setting. In this study, we analyzed the immunohistochemical expression of VEGF, microvessel density (MVD), and intratumoral DC infiltration in 132 surgically resected lung cancer specimens. We also evaluated the influence of these factors on their survival by a multivariate statistical analysis. VEGF expression was positively related to MVD (P = 0.0003) and negatively related to the degree of DC infiltration (P = 0.0232). A multivariate analysis also showed the VEGF expression, MVD, and DC infiltration to be independent prognostic factors. Moreover, we also accurately analyzed patient prognoses using the double stratification method for determining VEGF expression and DC infiltration. The patient group with a high VEGF expression/low DC infiltration showed a worse prognosis (P < 0.0001), whereas the group with a low VEGF expression/high DC infiltration had a better prognosis (P = 0.0001).

INTRODUCTION
Angiogenesis is an essential process required for the growth and metastatic ability of solid tumors (1, 2). A correlation between tumor angiogenesis and prognosis has been reported in various malignant tumors (3–5), including lung cancer (6, 7). VEGF plays a pivotal role in the development of neovascularization (8). Five VEGF isoforms (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206) have thus far been detected, and VEGF165 is reported to be the most abundant isoform in all human tissues. Several studies on solid tumors, including lung cancer, have demonstrated a correlation between the expression of VEGF and angiogenesis, and both factors are also important for assessing prognosis (9–12).

On the other hand, DCs are the most effective antigen-presenting cells for inducing primary immune responses to carcinoma (13). The density of DCs in tumor tissue has been reported to correlate with prognosis in certain human carcinomas (14–16). Previous studies have also shown a high DC infiltration in lung cancer to be related to a good prognosis (17), and the efficacy of immunotherapy using DCs has also been demonstrated (18–20).

Recently, several studies have indicated that VEGF inhibits both the maturation and function of DCs in vitro and in vivo (21–23). VEGF is thus suggested to be associated with not only an enhancement of angiogenesis but also a decline of local immune responses in tumors. On the basis of the findings of basic research, we hypothesized that the highly VEGF-expressing tumors suppress DC infiltration and contribute to poor prognosis. In this study, we analyzed the relationships among VEGF expression, MVD, and DC infiltration in NSCLC using immunohistochemical staining and also evaluated the influence of these parameters on the prognoses of patients with NSCLC.

MATERIALS AND METHODS
Patients’ Characteristics. Tumor specimens obtained from 132 patients with primary NSCLC undergoing surgical treatment at the Department of Surgery II, Kyushu University Hospital (Fukuoka, Japan), between 1989 and 1993 were studied. The specimens were taken from 93 men and 39 women with a median age of 68 years (range, 35–86 years). These tumors consisted of 65 adenocarcinomas, 54 squamous cell carcinomas, 12 large cell carcinomas, and 1 adenosquamous cell carcinoma. These included 34 well-differentiated, 63 moderately differentiated, 29 poorly differentiated, and 6 undifferentiated carcinomas. The patients were staged according to the surgical and pathological findings based on the guidelines described in the American Joint Committee on Cancer Staging Manual (24). Sixty-seven patients were determined to be stage I, 23 were stage II, 33 were stage III, and 9 were stage IV.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; DC, dendritic cell; MVD, microvessel density; NSCLC, non-small cell lung cancer; HPF, high power field.

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Immunohistochemistry for VEGF and Tumor-associated Vessels. Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan; Ref. 25). Resected tissue specimens were fixed in formalin, embedded in paraffin, cut into 4-μm serial sections, and then mounted on glass slides coated with poly-L-lysine. The slides were deparaffinized with xylene, dehydrated in graded alcohol, and incubated with 0.3% H₂O₂ solution in methanol for 30 min to block endogenous peroxidase. After washing three times with PBS, the slides were incubated in 10% normal goat serum for the immunohistochemical staining of VEGF or in 3% nonfat milk for the immunohistochemical staining of MVD to block nonspecific background staining. Next, the sections were incubated in anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; dilution, 1:100) or anti-factor VIII polyclonal antibody for endothelial surface marker (DAKO, Copenhagen, Denmark; dilution, 1:200) for 60 min at room temperature. This VEGF antibody recognizes the 121, 165, and 189 amino acid isoforms of human VEGF. After washing three times with PBS, the sections were incubated with streptavidin-biotinylated-peroxidase complex for 30 min and then washed once more with PBS three times. The sections were visualized by incubation with 3,3′-diaminobenzidine solution (0.03% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine) and counterstained with hematoxylin. Negative controls were carried out by substituting a normal rabbit IgG for primary antibody.

VEGF Expression and MVD. A histological analysis was performed by two investigators (N. I. and M. I.) simultaneously using a double-headed light microscope without knowledge of the patient’s clinical record. For the VEGF study, we selected the fields with the highest amount of VEGF expression in the specimen. The VEGF expression was evaluated according to the method described by Mattern et al. (26). Briefly, the intensity of the staining was graded according to a semiquantitative scale of 0–3 (scale a): 0, negative staining; 1, weak staining; 2, intermediate staining; 3, strong staining. The ratio of the positive cells was also graded semiquantitatively according to the positive cell percentage of 1000 cells counted (scale b): 0, no positive cell; 1, 1–25% positive cells; 2, 26–50% positive cells; 3, >50% positive cells. Finally, VEGF expression was
scored as a sum of (scale a: intensity) + (scale b: positive cell ratio) that reached a maximum score of 6. A histological analysis was performed twice in the same manner, and we used the average score as an index of VEGF expression in each patient.

MVD was evaluated in the most intense vascularization area in the tumor stroma, and 20 microscopic fields in total were selected to be examined in each tumor at ×100. The microvessel images were visualized on a computer display through a color video camera module (PVM-1454Q; Sony, Tokyo, Japan) and color image freezer (HC-1000; FUJI, Tokyo, Japan) and then were counted using the NIH image software package (version 1.61; Ref. 27). The vessels were identified based on the criteria proposed by Weidner et al. (3). The average of the 5 highest counts of 20 was used as the final MVD score.

**Immunohistochemistry for DCs.** Immunohistochemical staining with anti-S-100 antibody was performed to detect the DCs. The sections were deparaffinized and dehydrated and then incubated with 0.1% trypsin solution at 37°C for 30 min to unmask any cell surface antigen. After blocking endogenous peroxidase for 20 min, the sections were incubated overnight at 4°C in a moist chamber with anti-S-100 polyclonal antibody (Novocastra, Newcastle, United Kingdom; dilution, 1:150). The sections were incubated with the second antibody for 30 min and avidin-biotinylated peroxidase complex for 30 min. Finally, the sections were incubated with 3,3′-diaminobenzidine solution and counterstained with hematoxylin.

The number of S-100 protein-positive cells infiltrating the tumor nests was counted in 10 high power fields (×400) at random under the double-headed light microscopy by two investigators (N. I. and T. M.) simultaneously without any knowledge of the patient’s clinical records, and the mean value was calculated.

**Statistical Analysis.** Statview 5.0J statistical software (Abacus Concepts, Inc., Berkeley, CA) was used for all analyses. The χ² test and Fisher’s exact probability test were used to assess the correlation between immunoreactivity and clinicopathological factors. An evaluation of differences between the groups in VEGF expression, DC infiltration, and MVD was performed using the Mann-Whitney U test. The survival curves were generalized using the Kaplan-Meier method, and the prognoses were compared by log-rank test. A multivariate analysis of correlation between the factors and prognosis was performed using the Cox proportional hazard model. \( P < 0.05 \) was considered to be statistically significant, based on both univariate and multivariate analyses.

**RESULTS**

**Correlation of VEGF Expression Intensity and Clinical Parameters.** VEGF was mainly localized in the cytoplasm of the carcinoma cells (Fig. 1A). One hundred and twenty-five of the 132 (94.7%) samples were positive for VEGF while demonstrating various grades. Only seven samples were negative and graded as 0 (Fig. 1B). The distribution of the VEGF expression scores in all samples is shown as a histogram in Fig. 2. We classified the samples into two groups based on the median value consisting of a low VEGF group (0–4.5 scores, \( n = 65 \)) and a high VEGF group (≥5 scores, \( n = 67 \)). Table 1 shows the relationship between the VEGF expression intensity and clinicopathological factors. The VEGF expression intensity was significantly related to the pathological stage. The percentage of the high VEGF expression group was 40% (36 of 90) in stage I + II and 69% (29 of 42) in stage III + IV, and the difference was statistically significant (\( P = 0.0016 \)). However, no significant differences were observed between the other factors (age, sex, smoking history, histology, or differentiation) and the VEGF expression intensity.

**Correlation of MVD and Clinical Parameters.** The microvessels were assessed in the most intensive areas of neovascularization in the tumor stroma by an immunohistochemical survey of factor VIII (Fig. 1C). The median value of MVD was 126, ranging from 0 to 540.7. In 3 of 132 cases, no factor VIII-positive cells were found in the specimens. The distribution of MVD in all samples was shown as a histogram in Fig. 2 and displayed normal distribution. All samples were classified into...
two groups based on the median value consisting of a low MVD group (0–126, n = 66), and a high MVD group (>126, n = 66). No significant differences were observed between the two MVD groups regarding any clinical parameters (Table 1).

Correlation between DC Infiltration and Clinical Parameters. DCs, detected as S-100 protein-positive cells, were immunohistochemically found in the nests of tumor tissue specimens (Fig. 1D). No dominant neutrophil infiltration was observed in any samples, thus suggesting that causative bacterial infection of DC infiltration did not occur in the tumor tissue. DC infiltration into tumor nests was found in 116 of 132 (88%) samples, and the mean number of infiltrating DCs was 19.5 ± 21.9 cells/HPF, ranging from 0 to 104 cells/HPF. The histogram of DC infiltration did not show a normal distribution pattern (Fig. 2). From the pattern of the histogram, we classified DC infiltration into two groups at 20 cells/HPF consisting of a low MVD (14.7 ± 2.1 cells/HPF, n = 66), and a high infiltra-

<table>
<thead>
<tr>
<th>Table 1 Relationship between VEGF expression, DC infiltration, MVD, and clinicopathological factors</th>
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<tbody>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Age ≤68</td>
</tr>
<tr>
<td>≥68</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Smoking history +</td>
</tr>
<tr>
<td>–</td>
</tr>
<tr>
<td>Histology</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Not adenocarcinoma</td>
</tr>
<tr>
<td>Differentiation</td>
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<tr>
<td>Well</td>
</tr>
<tr>
<td>Not well</td>
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<tr>
<td>Stage</td>
</tr>
<tr>
<td>I + II</td>
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<tr>
<td>III + IV</td>
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</tbody>
</table>

Fig. 3 Comparison of DC infiltration intensity (P = 0.0232; A) and microvessel density (P = 0.0003; B) between the patients who had a high versus low VEGF expression score. Bars, SE.
the high MVD group was 27.8%, and that of the low MVD group was 60.4% (P<0.0001; Fig. 4B). The 5-year survival rate of the high DC infiltration group was 63.1%, and that of the low DC infiltration group was 31.9% (P=0.0004; Fig. 4C).

According to a multivariate analysis, the VEGF expression intensity, MVD, DC infiltration, and clinicopathological factors were analyzed by a Cox proportional hazards model. As shown in Table 2, VEGF expression intensity, MVD, and DC infiltration intensity were significant prognostic factors in addition to the disease stage. These three factors all had independent prognostic meaning after correcting for other clinical parameters such as age, sex, smoking history, differentiation, histology, and pathological stage.

Moreover, we tried to identify subgroups with better or worse prognosis by double stratification for VEGF expression intensity and DC infiltration intensity. Fig. 5 shows the survival curves and the overall survival rates of the patients stratified into four groups according to the basis of high or low intense VEGF expression and a high or low intense DC infiltration. The 5-year survival rates were 14.5% for the group of patients with a high VEGF expression and low DC infiltration, 43.4% for that for patients with a high VEGF expression and high DC infiltration, 55.0% for that for patients with a low VEGF expression and low DC infiltration, and 74.4% for that for patients with a low VEGF expression and high DC infiltration. Clearly, the subgroup with a low VEGF expression and high DC infiltration showed a significantly higher survival rate compared with the other three groups (P<0.0001). On the other hand, the subgroup with a high VEGF expression and low DC infiltration had a worse prognosis (P<0.0001).

**DISCUSSION**

Angiogenesis plays an important role in the growth, metastasis, and progression of solid tumors (1). The process of angiogenesis is thought to consist of a cascade of linked and sequential steps: dissolution of vascular basal membrane, chemotaxis, and a proliferation of vascular endothelial cells. Numerous promoters of angiogenesis have been identified. Among these angiogenic factors, positive regulators include members of VEGF, basic fibroblast growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and transforming growth factor-α. The negative regulators include angiotatin, endostatin, and IFN-inducible protein-10. These factors are produced by tumors or host cells such as macrophages. Among these factors, VEGF plays a crucial role in the control of angiogenesis, be-

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**Table 2** Multivariate analysis for overall survival based on the Cox proportional hazards model

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Hazard ratio</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&gt;68 yr)</td>
<td>1.547</td>
<td>0.932–2.560</td>
<td>0.0899</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>1.120</td>
<td>0.526–2.388</td>
<td>0.7685</td>
</tr>
<tr>
<td>Smoking history (+)</td>
<td>1.567</td>
<td>0.695–3.535</td>
<td>0.2790</td>
</tr>
<tr>
<td>Differentiation (not well)</td>
<td>1.936</td>
<td>0.967–3.879</td>
<td>0.0623</td>
</tr>
<tr>
<td>Histology (adenocarcinoma)</td>
<td>0.965</td>
<td>0.535–1.742</td>
<td>0.9066</td>
</tr>
<tr>
<td>Stage (III + IV)</td>
<td>2.855</td>
<td>1.649–4.940</td>
<td>0.0002</td>
</tr>
<tr>
<td>VEGF (high)</td>
<td>2.327</td>
<td>1.260–4.262</td>
<td>0.0069</td>
</tr>
<tr>
<td>DC infiltration (high)</td>
<td>0.520</td>
<td>0.290–0.932</td>
<td>0.0280</td>
</tr>
<tr>
<td>MVD (high)</td>
<td>3.162</td>
<td>1.882–5.313</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
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*CI, confidence interval.
cause VEGF is specific for the proliferation of endothelial cells and is closely associated with the angiogenesis of normal blood vessels to morbid blood vessels (28). VEGF is a M, 34,000–46,000 heparin-binding glycoprotein produced by either tumor cells or normal cells such as pituicytes, smooth muscle cells, macrophages, alveolar cells, and hepatocytes. In fact, the vascular and bronchial smooth muscle in our specimens showed weak staining for anti-VEGF antibody immunohistochemically. Tumor blood vessel formation is indispensable for solid tumors to develop beyond the microscopic stage. It is reported that most cancers including lung cancer produce VEGF, and that the intense VEGF expression is seen in cancers in which the tumor blood vessels are rich. Intense tumor neovascularization is associated with a high degree of malignancy (8).

Compared with cancers originating from other organs, lung cancer has varied histological and biological characteristics, regardless of its histological phenotype. Its prognosis is, in general, poor; the postoperative recurrence rate is high. In addition, hematogenous metastasis tends to already be recognized at the time of diagnosis in more than half of all cases. Therefore, angiogenesis, which is closely related to both invasion and the metastasis of cancer, would be important as an indicator of the biologically malignant characteristic of lung cancer. In the present study, we analyzed the relationship between the VEGF expression by tumor cells and MVD in tumor stroma in NSCLC by immunohistochemical staining and showed a significantly positive correlation between the VEGF expression and MVD. High VEGF-expressing tumors demonstrated a high MVD in this study, as reported previously (11, 12), and this finding supports the hypothesis that VEGF is one of the most important factors related to an increase in MVD. Furthermore, a high VEGF expression and high MVD in NSCLC were related to a worse prognosis than that related to low VEGF expression and low MVD, as indicated by a multivariate analysis, thus suggesting VEGF and MVD to be pivotal prognostic factors. On the other hand, a significant correlation between the prognosis and the number of immune cells in tumor tissues was reported (29–31). T cells are potentially important factors for suppressing the growth of tumors that escape the immune system (32). Clinically diagnosed cancers are the outcome of malignant cells that escape the immune defense mechanism and may suppress the state of the host immune system to survive and proliferate. The activation of the T cell begins by recognition of an antigen peptide presented by antigen-presenting cells. DCs have the most potent capacity to activate immature T cells, thus resulting in the initiation of an antigen-specific immune response and strong expression of HLA-DR and S-100 protein (33, 34). Several studies have indicated a correlation between DC infiltration and the prognosis of patients with malignant neoplasms (14–16), including lung cancer (17). In the present immunohistochemical study, a multivariate analysis also suggested that a high DC infiltration was significantly related to a better prognosis.

It has been reported that VEGF can inhibit DCs maturation in vitro, block DCs development, and decrease the number of DCs in vivo (21–22), whereas anti-VEGF antibody increased the number and function of DCs in vivo (23). These results of basic research closely correlate with our observation of clinical samples. Taken together, this inverse correlation might be attributable to the DC maturation inhibition by tumor-derived VEGF function. VEGF is supposed to deeply influence tumor growth, metastasis, and invasion by inhibiting the DC-mediated immune response. However, it remains to be elucidated whether VEGF inhibits DC infiltration in clinical human cancer tissue. This is the first report to show the inverse correlation between the VEGF expression intensity and DC infiltration in surgically resected lung cancer specimens. A similar inverse correlation was also reported in gastric cancer specimens (35). According to these findings, VEGF seems to clinically act as an inhibitor to DC function, as confirmed previously by basic research. If this is true, anti-VEGF therapy appears to be a promising cancer treatment for restoration of DC function in combination with suppression of tumor-associated angiogenesis. Furthermore, we also found each factor, i.e., the VEGF expression intensity or DC infiltration intensity, to be an independent prognostic factor based on a multivariate analysis. This finding led us to predict the prognosis of lung cancer patients precisely based on these two factors. As shown in Fig. 5, we clarified that a high VEGF expression/low DC infiltration is a poor prognostic factor. In our study, it was immunohistochemically shown that tumor tissues with a high VEGF expression had comparatively less DC infiltration in NSCLC, thus suggesting that VEGF may inhibit or control DCs, as suggested in both in vitro and in vivo experiments. Furthermore, high VEGF expression and low DC infiltration, in combination, was also found to be significantly related to a poor prognosis.

In conclusion, the VEGF expression and DC infiltration were inversely correlated in tumor specimens of NSCLC. We can predict the patient prognosis by a double stratification analysis of both the VEGF expression and DC infiltration.

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