Expression of Vascular Endothelial Growth Factors A, B, C, and D and Their Relationships to Lymph Node Status in Lung Adenocarcinoma

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ABSTRACT

Vascular endothelial growth factors (VEGFs) C and D are novel members of the VEGF family that show some selectivity toward lymphatic endothelial cells. Recent studies suggest that VEGF-C may be involved in lymphangiogenesis and spread of cancer cells via lymphatic vessels. However, whether other VEGF family members play a role in lymph node metastasis is largely unknown. The aim of the present study was to explore whether expressions of VEGF-A, VEGF-B, VEGF-C, and VEGF-D are correlated with lymph node status in lung adenocarcinoma. Total RNA was isolated from 60 surgical specimens of lung adenocarcinoma with (n = 27) or without (n = 33) lymph node metastasis. The relative mRNA abundance of VEGF-A, VEGF-B, VEGF-C, and VEGF-D was measured by real-time reverse transcription-PCR analysis based on TaqMan fluorescence methodology. We found that, as single factors, expression of none of the four VEGF family members clearly correlated with the presence of lymph node metastasis. The only tendency noted was for higher VEGF-B and VEGF-C and lower VEGF-D levels in the node-positive group. However, two-way scatterplot analysis revealed that tumors with lymph node metastasis were associated with a pattern of low VEGF-D and high VEGF-A, VEGF-B, or VEGF-C, such that the ratios of VEGF-D:VEGF-A, VEGF-D:VEGF-B, or VEGF-D:VEGF-C were significantly lower in the node-positive group. Strikingly, none of the 11 tumors with high VEGF-D levels metastasized to lymph nodes. Furthermore, a low VEGF-D:VEGF-C ratio correlated with the presence of lymphatic invasion, and six of seven tumors with a pattern of very high expression of VEGF-C and low expression of VEGF-D displayed lymph vessel invasion that extended along the bronchovascular tree beyond the main tumor. Finally, levels of VEGF-A, but not VEGF-B or VEGF-C, were higher in tumors with large nodal metastasis (≥1 cm) than in those with small (<1 cm) nodal metastasis. These results support the hypothesis that two VEGF family members are involved in lymph node metastasis at two distinct steps; VEGF-C facilitates entry of cancer cells into the lymph vasculature, whereas VEGF-A promotes the growth of metastatic tumor through angiogenesis. The results also suggest that the balance between VEGF-C and VEGF-D could be important rather than the level of VEGF-C alone. Whether a low VEGF-D level plays a causative role in lymph node metastasis requires further investigation.

INTRODUCTION

The VEGF family is a group of growth factors that regulate the growth of endothelial cells (1, 2). The founding member, VEGF-A, plays essential roles in vasculogenesis and angiogenesis (3). Its crucial role in tumor angiogenesis and blood-borne metastasis has been documented in a variety of cancers (4). Although expression of VEGF-A in cancer has been studied extensively, the roles of other VEGF family members, i.e., VEGF-B, VEGF-C, and VEGF-D, in tumor angiogenesis and metastasis are poorly understood (5).

VEGF-C (6–9) was initially identified as a ligand of VEGFR-3, which at the time was an “orphan” receptor that showed sequence similarity to VEGFR-1 and VEGFR-2. Because expression of VEGFR-3 is largely restricted to lymphatic endothelium (10–12), the major function of VEGF-C appears to be the regulation of lymphatic vessel growth. Transgenic mice that overexpress VEGF-C in keratinocytes develop numerous dilated lymphatic vessels in the skin (13). Recent studies suggest that VEGF-C may promote spread of cancer cells through lymphatic channels (14, 15). VEGF-D was isolated as a fos-inducible factor from mouse skin fibroblasts (16) and through database searches for sequences that show homology to VEGF-C (17, 18). Because of their sequence similarity, VEGF-C and VEGF-D are thought to have similar biological functions.

In the present study, we studied the expression of VEGF-C and VEGF-D in lung adenocarcinoma and investigated its relationship to lymph node metastasis, one of the most important prognostic factors in lung cancer (19, 20). We also studied the...
expression of VEGF-A and VEGF-B. VEGF-A appears to be involved in lymph node metastasis of lung and colorectal cancers (21–23); the significance of VEGF-B expression in tumors is still unclear (5, 24, 25).

**MATERIALS AND METHODS**

**Tissue Samples and Patients.** Tissue samples were obtained from 60 primary lung adenocarcinomas that were resected at the National Cancer Center Hospital in 1997 and 1998. Normal lung tissues were obtained from nontumorous portions of the lobectomy specimens that were distant from the primary tumor. These control specimens were taken from lung tissues histologically confirmed to be without fibrosis, inflammation, or emphysema. Tissues were quickly frozen in liquid nitrogen until RNA extraction. Lymph node status was determined by routine pathological examination of dissected pulmonary hilar and mediastinal lymph nodes, as well as intrapulmonary lymph nodes. At least six dissected lymph nodes were histologically examined in all of the node-negative cases. Because some of the tumors without lymph node metastasis were putative noninvasive adenocarcinomas, as described by Noguchi et al. (26), these tumors were analyzed as a separate group. The clinicopathological characteristics of the patients are summarized in Table 1. The stage of the disease was based on the Tumor-Node-Metastasis classification of the Union Internationale Contre le Cancer (27).

**Cell Lines and Culture.** Lung adenocarcinoma cell lines A549, RELF-LC-MS, VMRC-LCD, and ABC-1 were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan), and HLC-1 and LC-2/ad were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The lung adenocarcinoma cell line L-27 was established in our laboratory. All cell lines were cultured in DMEM supplemented with 10% FCS, glutamine, and antibiotics in a humidified atmosphere of 5% CO2 and 95% air.

**RNA Extraction.** Total RNA was isolated using a RNeasy kit (Qiagen). The purity and concentration of RNA were determined by spectrophotometry at 260 nm. The quality of RNA was checked by the electrophoresis of 2–3-μg samples in a 1% agarose gel, staining with ethidium bromide, and examining the 28S and 18S rRNA bands on a UV transilluminator. No significant degradation was observed in any RNA samples.

**Real-Time RT-PCR.** The quantitation of relative mRNA abundance was carried out using a real-time fluorescence detection method using TaqMan chemistry (28). This method has been used in several recent papers to measure gene expression (29–32). Briefly, 50 ng of total RNA were reverse transcribed, and the gene of interest was PCR amplified with rTth DNA polymerase using gene-specific primers in a single-tube, single-enzyme system. During PCR amplification, the 5′-3′ nuclease activity of the rTth DNA polymerase releases a TaqMan probe that hybridized to amplified sequences. The TaqMan probe contains a reporter dye (FAM) at the 5′ end and quencher dye (TAMRA) at the 3′ end. Cleavage of the probe by rTth DNA polymerase separates the reporter from the quencher. The resulting increase of fluorescence signal can be detected by the laser detector of the ABI Model 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Urayasu, Chiba). In this system, the cycle at which the fluorescent intensity first exceeds a threshold value (Ct) is determined for each sample. By running serial dilutions of a reference sample, a standard curve is generated in each experiment. The target message of the unknown sample is then determined by measuring its Ct and by using the standard curve. The linearity of the standard curve ensures that the starting quantity of the target molecule is inversely proportional to its Ct. The reference RNA samples used to generate standard curves were obtained from a lung adenocarcinoma cell line, L-27, for VEGF-A, VEGF-B, and VEGF-C, and a neuroblastoma cell line, NB39 nu, for VEGF-D. All experiments were performed in duplicate, and the mean values were calculated. The results were normalized for 18S rRNA, the abundance of which was also determined by TaqMan RT-PCR. The results were expressed in arbitrary units. Negative controls lacking template RNA were always included in each experiment.

The following oligonucleotides were used for real-time RT-PCR: (a) forward VEGF-A primer, 5′-CTTGCCCTTGCT-GCTCTACC-3′; reverse VEGF-A primer, 5′-CAGCAAGGAT-GGCTTTGAAG-3′; VEGF-A probe, 5′-FAM AGTCTACACCCAGCT TAMARA-3′; (b) forward VEGF-B primer, 5′-AGCACCAAGTCCCGAT-3′; reverse VEGF-B primer, 5′-ATGCTGCTACAGCAGCT-3′; VEGF-B probe, 5′-FAM AGATCCCTCATGATCGTGACT-3′; (c) forward VEGF-C primer, 5′-TGGCGATGTCGTTCAA-3′; reverse VEGF-C primer, 5′-TGAACAGGTCTCTTCATCCAGC-3′; VEGF-C probe, 5′-FAM CAGCAGACTCCAGCT-3′; (d) forward VEGF-D primer, 5′-GATGGAATTGTCGCTGAG-3′; reverse VEGF-D primer, 5′-AGGCTTCTTCTATCAGCA-3′; VEGF-D probe, 5′-FAM AAGAACAGTTCAGACAG-3′.
ACG TAMRA-3'; and (e) forward 18S rRNA primer, 5'-CG-GCTACACATCCAAAGAA-3'; reverse 18S rRNA primer, 5'-GCTGGAATTACCGGCTGTC-3'; 18S rRNA probe, 5'-6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein TGCTGCGACACTTGCACCCT TAMRA-3'.

The primers for VEGF-A span exon 1 to exon 3 and thus detect all isoforms of VEGF-A. To distinguish different VEGF-A isoforms, a second set of primers were designed: a second forward VEGF-A primer, 5'-GAGTGCCCCACT-GAGGAGTC-3', and a second reverse VEGF-A primer, 5'-GCCTCGGCTTGTCACAT-3'. These primers spanned exon 3 to exon 8 and thus allowed discrimination between different isoforms of VEGF-A, i.e., VEGF-A121, VEGF-A165, VEGF-A189, and VEGF-A206, by the electrophoresis of PCR products in agarose gels.

The PCR amplification was performed using a 96-well optical tray and caps in a final reaction volume of 50 μl. The reaction mixture consisted of 200 nM each primer, 100 nM probe, 0.1 unit/μl T7 DNA polymerase, 300 μM each of dATP, dCTP, and dGTP, 600 μM dUTP, 3 mM manganese acetate, 0.01 unit/μl AmpErase uracil N-glycosylase, and 1× TaqMan buffer containing a reference dye. The RT-PCR cycle parameters were as follows: 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Primers and probes were designed using the computer program Primer Express (Perkin-Elmer), following the instructions of the manufacturer. Primers were chosen from sequences of different exons. Sequence specificity of the primers and probes was confirmed by homology searches through databases at National Center for Biotechnology Information using the computer program BLASTN. Additionally, we sequenced the PCR products and confirmed their identity with the target sequence. Primers and probes were purchased from Greiner Japan (Tokyo) and Perkin-Elmer Applied Systems, respectively.

Statistics. Kruskal-Wallis one-way ANOVA was first performed to decide whether there was a significant difference between different groups. If we observed statistical difference by this global analysis, the difference between two groups was determined by the Mann-Whitney U test. The correlation between clinicopathological parameters and levels of VEGF family members was evaluated by Spearman’s rank correlation coefficients. The correlation between the presence of lymph node metastasis and that of lymphatic invasion in the 60 cases of lung adenocarcinoma was determined by Fisher’s probability exact test. Calculation was performed using the computer program StatView (Abacus Concepts, Berkeley, CA). The results were considered significant if P < 0.05.

RESULTS

Expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D mRNA in Lung Adenocarcinoma, Normal Lung Tissue, and Lung Adenocarcinoma Cell Lines. Total RNA was extracted from 60 cases of lung adenocarcinoma, 9 historically normal samples of lung tissues that were distant from the primary tumor, and 7 lung adenocarcinoma cell lines. Relative expression levels of VEGF-A, VEGF-B, VEGF-C, and VEGF-D were quantitated using TaqMan fluorescent RT-PCR analysis. The results are shown in Figs. 1 and 2.

VEGF-A was expressed by all specimens, regardless of whether they were derived from tumors or nontumoral tissues. RT-PCR with the second set of VEGF-A primers showed that, in keeping with previous studies (21, 22), VEGF-A121 and VEGF-A165 were the predominant isoforms in lung adenocarcinoma and normal lung tissues (data not shown). VEGF-B was also expressed by all tumors and normal lung tissues. Because of the great variations within each group, however, we could not find any significant differences in VEGF-A and VEGF-B levels between groups. Nevertheless, VEGF-B levels appeared to be higher in the node-positive group than in the node-negative group.

Expression patterns of VEGF-C and VEGF-D were quite different from those of VEGF-A and VEGF-B. Normal lung tissues expressed both VEGF-C and VEGF-D at much higher levels than did tumor tissues (P < 0.01 and P < 0.002, respectively). Comparison between different tumor groups showed that VEGF-C levels tended to be higher in the node-positive group than in the node-negative group, whereas VEGF-D levels tended to be lower in the node-positive group than in the node-negative group. Interestingly, VEGF-D levels seemed to decrease in the following order: normal lung tissue, noninvasive...
VEGFs and Lymph Node Metastasis in Lung Adenocarcinoma

Expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D mRNA in seven lung adenocarcinoma cell lines. All of the seven adenocarcinoma cell lines expressed VEGF-A and VEGF-B at levels comparable with those of primary tumors. VEGF-C was expressed at variable levels in the seven cell lines: at a high level in ABC-1, L-27, LC-2/ad, and RERF-LC-MS; at a modest level in A549; and at very low levels in HLC-1 and VMRC-LCD. As in primary tumors, expression levels of VEGF-D were very low in all of the seven cell lines.

We then checked whether expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D was correlated with other clinicopathological parameters, such as tumor size, T status, and pathological stage. As shown in Table 2, we noted a correlation between VEGF-B and pathological stage (P = 0.03) and a negative correlation between VEGF-D and tumor size (P = 0.002). VEGF-D levels were very low (<2.0) in all of the tumors >3.0 cm in diameter. We did not find any other correlation between expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D and the above clinicopathological parameters.

Lymph Node Metastasis Is Correlated with Low Ratios of VEGF-D versus VEGF-A, VEGF-B, and VEGF-C. As a single factor, expression of none of the VEGF family members investigated clearly correlated with lymph node metastasis. However, because the VEGF family members share a receptor system (2), i.e., VEGF-A binds to VEGFR-1 and VEGFR-2, VEGF-B to VEGFR-1, and VEGF-C and VEGF-D to VEGFR-2 and VEGFR-3, analysis of combinations of two factors may reveal an association better than analysis of a single factor. Therefore, we performed two-way scatterplot analyses such that expression levels of two VEGF family members can be seen on the same diagram. Of the six possible combinations, we observed an interesting pattern when VEGF-D was combined with VEGF-A, VEGF-B, or VEGF-C (Fig. 3). All of the tumors with lymph node metastasis showed very low levels of VEGF-D (<2.0) and relatively high levels of VEGF-A, VEGF-B, and VEGF-C. Strikingly, lymph node metastasis occurred in 9 of 10 tumors with high VEGF-C (>2.0) and low VEGF-D (<2.0) levels. These characteristic expression patterns were reflected in lower ratios of VEGF-D/VEGF-A, VEGF-D/VEGF-B, and VEGF-D/VEGF-C in node-positive tumors (Fig. 4; P = 0.042, 0.027, and 0.011, respectively).

Although the large majority of tumors showed low levels and at very low levels in HLC-1 and VMRC-LCD. As in the primary tumors, expression levels of VEGF-D were very low in all of the seven cell lines.

The correlation between VEGF-A, VEGF-B, VEGF-C, and VEGF-D mRNA levels and T factor, p stage, and tumor size is shown in Table 2.

Table 2: Correlation between VEGF-A, VEGF-B, VEGF-C, and VEGF-D mRNA levels and T factor, p stage, and tumor size.

<table>
<thead>
<tr>
<th>T Factor</th>
<th>VEGF-A</th>
<th>VEGF-B</th>
<th>VEGF-C</th>
<th>VEGF-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>3.2 ± 2.6</td>
<td>4.6 ± 2.4</td>
<td>1.5 ± 1.3</td>
<td>1.9 ± 2.4</td>
</tr>
<tr>
<td>T2</td>
<td>3.0 ± 2.6</td>
<td>4.5 ± 2.4</td>
<td>1.8 ± 1.3</td>
<td>1.9 ± 2.4</td>
</tr>
<tr>
<td>T3</td>
<td>2.5 ± 2.6</td>
<td>6.5 ± 2.1</td>
<td>1.7 ± 1.1</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>T4</td>
<td>3.0 ± 2.6</td>
<td>4.3 ± 2.2</td>
<td>1.3 ± 1.3</td>
<td>2.4 ± 2.7</td>
</tr>
<tr>
<td>p stage</td>
<td>P = 0.20</td>
<td>P = 0.03</td>
<td>P = 0.19</td>
<td>P = 0.15</td>
</tr>
<tr>
<td>nb</td>
<td>1.0 ± 2.6</td>
<td>4.0 ± 2.1</td>
<td>1.7 ± 1.3</td>
<td>2.4 ± 2.7</td>
</tr>
<tr>
<td>ib</td>
<td>4.5 ± 2.6</td>
<td>6.5 ± 2.1</td>
<td>1.7 ± 1.3</td>
<td>2.4 ± 2.7</td>
</tr>
<tr>
<td>ii</td>
<td>6.2 ± 2.6</td>
<td>5.5 ± 2.1</td>
<td>1.7 ± 1.3</td>
<td>2.4 ± 2.7</td>
</tr>
<tr>
<td>iii</td>
<td>3.2 ± 2.6</td>
<td>5.4 ± 2.1</td>
<td>1.7 ± 1.3</td>
<td>2.4 ± 2.7</td>
</tr>
<tr>
<td>Tumor size</td>
<td>P = 0.21</td>
<td>P = 0.72</td>
<td>P = 0.62</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>1 ≤ r &lt; 2 cm</td>
<td>2.7 ± 2.2</td>
<td>4.4 ± 2.4</td>
<td>1.3 ± 1.1</td>
<td>2.0 ± 2.0</td>
</tr>
<tr>
<td>2 ≤ r &lt; 3 cm</td>
<td>3.4 ± 2.0</td>
<td>4.5 ± 1.6</td>
<td>1.6 ± 1.3</td>
<td>1.9 ± 2.6</td>
</tr>
<tr>
<td>3 ≤ r &lt; 4 cm</td>
<td>4.9 ± 2.2</td>
<td>5.7 ± 2.9</td>
<td>1.5 ± 1.1</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>4 ≤ r &lt; 5 cm</td>
<td>5.6 ± 2.2</td>
<td>4.5 ± 2.4</td>
<td>0.5 ± 0.5</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

* VEGF-B was correlated with pathological stage, whereas VEGF-D was negatively correlated with tumor size.

b p, pathological.

Fig. 2 Expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D mRNA in seven lung adenocarcinoma cell lines. All of the seven adenocarcinoma cell lines expressed VEGF-A and VEGF-B at levels comparable with those of primary tumors. VEGF-C was expressed at variable levels in the seven cell lines: at a high level in ABC-1, L-27, LC-2/ad, and RERF-LC-MS; at a modest level in A549; and at a very low level in HLC-1 and VMRC-LCD. As in primary tumors, expression levels of VEGF-D were very low in all of the seven cell lines. The results were corrected for 18S rRNA and were expressed in arbitrary units. 1, A549; 2, HLC-1; 3, ABC-1; 4, L-27; 5, LC-2/ad; 6, RELF-LC-MS; 7, VMRC-LCD.
of VEGF-D (<2.0), 11 tumors showed relatively high levels of VEGF-D (Fig. 3). Interestingly, none of these 11 tumors showed lymph node metastasis. These 11 tumors were also characterized by relatively low VEGF-A expression, whereas expression levels of VEGF-C and VEGF-D varied. Thus, these 11 tumors appeared to constitute a separate group. Among the 49 tumors with low VEGF-D levels (<2.0), node-positive cases showed higher VEGF-B ($P = 0.017$) and VEGF-C ($P = 0.007$) levels than node-negative cases.

**Lymphatic Invasion of Cancer Cells Is Correlated with a Low VEGF-D:VEGF-C Ratio.** Next, we asked whether lymphatic invasion of cancer cells is correlated with expression of VEGF-C or other VEGF family members. Fig. 5 shows mRNA levels of the four VEGF family members in tumors with ($n = 41$) or without ($n = 19$) lymphatic invasion. We found that VEGF-C levels tended to be higher ($P = 0.053$) and VEGF-D levels were lower ($P = 0.006$) in tumors with lymphatic invasion than in those without lymphatic invasion.
Although the large majority of node-positive tumors (93%; 25 of 27) showed lymphatic invasion of cancer cells, invasion was found in 59% (16 of 27) of node-negative tumors. Thus, the presence of lymph node metastasis was correlated with lymphatic invasion of cancer cells (Fisher’s exact probability test, \( P < 0.009 \)). We divided node-negative tumors into two groups on the basis of the presence or absence of lymphatic invasion and determined whether node-negative tumors with lymphatic invasion had characteristics intermediate between those of node-positive tumors and node-negative tumors without lymphatic invasion. Scatterplot analysis confirmed that node-negative tumors with lymphatic invasion showed a relatively high VEGF-C and low VEGF-D expression pattern as compared with node-negative tumors without lymphatic invasion (Fig. 6). This was reflected in the lower VEGF-D:VEGF-C ratio (\( P < 0.001 \)) in this group (Fig. 7). We performed similar analyses using other combinations of VEGF family members. We found a marginally significant difference in VEGF-D:VEGF-B ratio between the two groups (\( P = 0.037 \)), but it was not as clear as for VEGF-D:VEGF-C.

Among those tumors with lymphatic invasion, seven cases showed lymphatic invasion that extended along the bronchovascular tree beyond the main tumor, a histological feature resembling “lymphangeal carcinomatosis.” Interestingly, six of seven tumors with this feature exhibited an expression pattern characterized by very high VEGF-C (>2.0) and low VEGF-D (<2.0).

**VEGF-A Levels Are Correlated with Bulky Lymph Node Metastasis.** Metastasis involves a cascade of events that include: (a) invasion of cancer cells into blood or lymph vasculature; (b) suppression of anoikis; (c) evasion from immune surveillance; and (d) local growth at metastatic sites (4). We reasoned that VEGF-A may be involved in the growth of metastatic tumor cells through induction of angiogenesis in the lymph nodes. To confirm this, we subdivided the node-positive group into two subgroups, one with bulky lymph node metastasis (\( \geq 1 \) cm in diameter), and the other group with small lymph node metastasis (<1 cm in diameter). Then we compared the expression levels of VEGF family members in the different groups. As shown in Fig. 8, VEGF-A levels were higher in tumors with bulky lymph node metastasis than in tumors with small lymph node metastasis. VEGF-A levels in tumors with small lymph node metastasis were very low and appeared to be even lower than in node-negative tumors. Thus, VEGF-A is likely to be involved in the growth of tumor at metastatic sites. We compared levels of other VEGF family members between the different groups, but we did not find any significant correlations (Fig. 8).
DISCUSSION

In the present study, we investigated whether expression of four VEGF family members, VEGF-A, VEGF-B, VEGF-C, and VEGF-D, is associated with lymph node status in lung adenocarcinoma. Our main hypothesis was that VEGF-C may contribute to lymph node metastasis by facilitating the entry of cancer cells into lymphatic vessels. We found, however, that as a single factor, none of the four VEGF family members correlated with lymph node metastasis. Surprisingly, both VEGF-C and VEGF-D mRNA levels were much higher in normal lung tissue than in tumorous tissue. Although this was rather unexpected, high levels of VEGF-C (7, 8, 11, 33) and VEGF-D (17, 18) have been described in adult lung tissues. The functional roles of these growth factors in adult lung is unknown, but they may be involved in homeostasis of lung vasculature, which may perform specialized functions.

Despite this lack of correlation by single factor analysis, scatterplot analysis revealed that an expression pattern of high VEGF-C and low VEGF-D, or a low VEGF-D:VEGF-C ratio, was correlated with both lymph node metastasis and lymphatic invasion of cancer cells. Interestingly, those tumors that showed especially high levels of VEGF-C and low levels of VEGF-D displayed lymphatic invasion of cancer cells outside the main tumor, a situation somewhat resembling “lymphangeal carcinomatosis.” These results are consistent with recent reports that showed positive correlation of VEGF-C levels and lymph node metastasis in prostate and stomach cancer (14, 15). Because VEGF-D was not measured in these studies, however, it would be necessary to see whether analysis of the combination of VEGF-C and VEGF-D reveals similar findings in these cancers.

Our study showed that VEGF-D levels were down-regulated in the majority of tumor specimens as compared with normal tissue. However, a fraction of lung adenocarcinomas (11 of 60), especially those that were small in size (<3 cm in diameter), retained relatively high levels of VEGF-D mRNA. Interestingly, none of these 11 tumors metastasized to lymph nodes, although some of them expressed VEGF-C at a relatively high level. Whether this is attributable to a possible antimetastatic effect of VEGF-D, or whether the observation of no

Fig. 6 Scatterplot analysis showing VEGF-C and VEGF-D in different groups of lung adenocarcinoma subdivided on the basis of lymphatic invasion. The VEGF-D:VEGF-C ratio was lower in node-negative tumors with lymphatic invasion compared with those without lymphatic invasion. The VEGF-D:VEGF-B ratio was also lower in tumors with lymphatic invasion, but the difference was not as clear as that with VEGF-D:VEGF-C. 1, normal lung; 2, noninvasive adenocarcinoma; 3, node-negative lung adenocarcinoma without lymphatic invasion; 4, node-negative lung adenocarcinoma with lymphatic invasion; 5, node-positive lung adenocarcinoma.

Fig. 7 Ratios of VEGF-D:VEGF-A, VEGF-D:VEGF-B, and VEGF-D:VEGF-C in normal lung and in different groups of lung adenocarcinoma subdivided on the basis of lymphatic invasion. The VEGF-D:VEGF-C ratio was lower in node-negative tumors with lymphatic invasion compared with those without lymphatic invasion. The VEGF-D:VEGF-B ratio was also lower in tumors with lymphatic invasion, but the difference was not as clear as that with VEGF-D:VEGF-C. 1, normal lung; 2, noninvasive adenocarcinoma; 3, node-negative lung adenocarcinoma without lymphatic invasion; 4, node-negative lung adenocarcinoma with lymphatic invasion; 5, node-positive lung adenocarcinoma.
metastasis in the context of a high VEGF-D level represents a mere association, remains to be clarified.

Tumor cells must undergo multiple steps to establish metastasis (4). For lymph node metastasis to occur, tumor cells must: (a) enter lymphatic vessels; (b) suppress apoptosis during detachment from the matrix; (c) evade immune surveillance; and (d) turn on the angiogenic switch at metastatic sites. Previous studies found a positive correlation between VEGF-A expression and lymph node metastasis in lung and colorectal cancers (21–23). The reason for this correlation was not clear, but it is conceivable that VEGF-A may promote lymph node metastasis of cancer cells through induction of angiogenesis in the lymph nodes. Our observation that VEGF-A levels were higher in tumors with bulky lymph node metastasis than in those with small lymph node metastasis is consistent with this hypothesis. Because metastasis is considered to be a selective process (4), it is arguable that the characteristics of the metastatic cells with regard to VEGF expression may not be the same as those of cells in the primary tumors. However, this does not necessarily mean that the relevance of data obtained from primary tumors is questionable in terms of deducing or predicting the behavior of tumor cells at metastatic sites. A multitude of previous studies have shown that expression levels of given factors, including VEGF (34), are correlated with the presence of metastasis and/or disease recurrence.

Taken together, our data are consistent with the hypothesis that VEGF-A and VEGF-C promote lymph node metastasis by distinct mechanisms; the latter facilitates entry of tumor cells into the lymph vasculature, whereas the former stimulates tumor growth at metastatic sites through angiogenesis. VEGF-B appears to be involved in lymph node metastasis in the setting of low VEGF-D and in the progression of lung adenocarcinoma because it was associated with pathological stage. The precise roles of these VEGF family members in cancer progression and metastasis should be elucidated by further functional studies.

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