Significance of Vessel Count and Vascular Endothelial Growth Factor in Human Esophageal Carcinomas

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ABSTRACT

The purpose of this study was to determine the angiogenic profile of human esophageal carcinomas. The expression of vascular endothelial growth factor (VEGF) was examined in 6 esophageal carcinoma cell lines and 119 human esophageal carcinoma tissues by Northern blot analysis and immunohistochemistry, respectively. Immunohistochemistry using antibodies against CD34 (endothelial cell specific) was carried out on archival specimens, and microvessels were quantitated by counting vessels in a $\times$200 field in the most vascular area of the tumor. All of the cell lines constitutively expressed VEGF mRNA at various levels. A total of 71 of 119 (59.7%) tumors showed intense VEGF immunoreactivity in the cytoplasm of cancer cells. Vessel count was significantly higher in the VEGF-positive tumors than it was in the VEGF-negative tumors. VEGF expression correlated with the depth of tumor invasion, tumor stage, venous invasion, and lymphatic invasion. The survival rate of patients with high vessel density in the tumor was significantly worse than that of patients with low vessel density in the tumor. There was a tendency for poorer prognosis in the group with VEGF-positive tumors compared with that of the group with VEGF-negative tumors. Overall, these results suggest that VEGF is associated with tumor progression by stimulating angiogenesis in human esophageal carcinoma.

INTRODUCTION

Angiogenesis, which is essential for tumor growth and metastasis, depends on the production of angiogenic factors by tumor cells and normal cells (1, 2). Increased vascularity enhances the growth of primary neoplasms and provides a greater chance for hematogenous metastasis. Previous studies have shown that increased vascularity is associated with a worse prognosis in certain tumors, including breast (3), lung (4), prostate (5), cervical (6), and colon carcinomas (7) and melanoma (8). We have recently reported that gastric carcinoma cells expressed several angiogenic factors such as basic fibroblast growth factor (9), VEGF (10, 11), and IL-8 (12). However, vascularity in gastric carcinoma was most closely correlated with the expression of IL-8 (11, 12).

Esophageal carcinoma is one of the most common malignancies in the world and shows dramatic regional variations in incidence (13). Its growth is relatively fast, and patients with esophageal carcinoma generally have a worse prognosis than those with any other kind of gastrointestinal tumor (14). To elucidate better prognostic indicators, many researchers have investigated the clinicopathological features. However, little is known about the angiogenic profile in esophageal carcinoma.

VEGF is a $M_r$ 34,000–50,000 dimeric glycoprotein synthesized by both tumor cells and normal cells (15). It consists of four isoforms that have 121, 165, 189, or 206 amino acid residues (16, 17). All four forms induce mitogenesis of vascular endothelial cells and vascular permeabilization. Previous reports have shown that VEGF acts via the paracrine mechanism through specific receptors on the surface of endothelial cells, flt-1 and KDR (18, 19).

To determine whether VEGF plays a role in the progression of esophageal carcinomas, we examined the expression of VEGF in human esophageal carcinoma and correlated it with vessel density. We also attempted to compare these data with clinicopathological findings.

MATERIALS AND METHODS

Cell Cultures. Six cell lines established from human esophageal carcinomas (TE-3, well-differentiated squamous carcinoma; TE-2 and TE-5, poorly differentiated squamous carcinoma; TE-7, adenocarcinoma; and TE-8 and TE-12, moderately differentiated squamous carcinoma) were kindly provided by Dr. T. Nishida (Tohoku University School of Medicine, Sendai, Japan). Cells were maintained in RPMI 1640 (Nissui Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (M. A. Bioproducts, Inc., Walkersville, MD).

Patients and Tumor Specimens. Paraffin-embedded tumor specimens from 119 patients with esophageal carcinomas...
Table 1 Relationship between VEGF expression, vessel count, and clinicopathological features in human esophageal carcinomas

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of cases</th>
<th>VEGF positivity</th>
<th>Vessel count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological grade&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>35</td>
<td>68.6 (24/35)</td>
<td>42.9 ± 3.4</td>
</tr>
<tr>
<td>Moderate</td>
<td>67</td>
<td>55.2 (37/67)</td>
<td>43.5 ± 2.0</td>
</tr>
<tr>
<td>Poor</td>
<td>14</td>
<td>71.4 (10/14)</td>
<td>61.2 ± 5.6</td>
</tr>
<tr>
<td>Depth of invasion&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m and sm</td>
<td>53</td>
<td>35.8 (19/53)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.1 ± 2.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>mp and a</td>
<td>66</td>
<td>78.7 (52/66)</td>
<td>55.1 ± 2.9</td>
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<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–2</td>
<td>67</td>
<td>43.3 (29/67)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>41.7 ± 2.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 and 4</td>
<td>52</td>
<td>80.8 (42/52)</td>
<td>55.1 ± 3.4</td>
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<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>42</td>
<td>31.0 (13/42)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>39.9 ± 3.0&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Positive</td>
<td>77</td>
<td>75.3 (58/77)</td>
<td>51.7 ± 2.6</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>73</td>
<td>46.6 (34/73)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>46.4 ± 2.6</td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>80.4 (37/46)</td>
<td>49.4 ± 3.2</td>
</tr>
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</table>

<sup>a</sup> Classification was made according to the rules established by the Japanese Society for Esophageal Diseases (20).

<sup>b</sup> Mann-Whitney U test.

<sup>c</sup> Well, well-differentiated squamous carcinoma; moderate, moderately differentiated squamous carcinoma; poor, poorly differentiated squamous carcinoma.

<sup>d</sup> m, mucosa; sm, submucosa; mp, muscularis propria; a, adventitia.

<sup>e</sup> P < 0.01.

<sup>f</sup> P < 0.05.

who had undergone surgery at the Hiroshima University Hospital were studied using immunohistochemistry. Pathology reports and clinical histories at the time of surgery were reviewed to determine accurate staging. The majority of patients with invasive carcinoma (81 individuals) received adjuvant chemotherapy and/or radiation therapy.

The stage grouping and the histological classification were defined according to the criteria of the Japanese Society for Esophageal Disease (20). The patients ranged in age from 39–86 years (mean, 63.5 years); patients included 107 men and 12 women. Histological grades, tumor stages, and the depth of invasion are shown in Table 1.

**Northern Blot Analysis.** Polyadenylated mRNA was extracted from esophageal carcinoma cell lines using the Fast-Track mRNA isolation kit (Invitrogen Co., San Diego, CA). mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 A to a GeneScreen nylon membrane (DuPont New England Nuclear, Boston, MA), and UV cross-linked with 120,000 mJ/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously (21). Nylon filters were washed at 65°C with 30 mM NaCl, 3 mM sodium citrate (pH 7.2), and 0.1% SDS (w/v).

The cDNA probes used in these analyses were a 1.3-kb fragment corresponding to rat glyceraldehyde 3-phosphate dehydrogenase (Clontech, Palo Alto, CA) and a 0.7-kb fragment corresponding to human VEGF (kindly provided by Dr. M. Shibuya, University of Tokyo, Tokyo, Japan). Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled using the random primer technique with 32P-labeled deoxyribonucleotide triphosphates (22).

**Immunohistochemical Staining.** Consecutive 4-μm sections were cut from each study block. Sections were immunostained for VEGF and CD34 (specific for endothelial cells). Immunohistochemical staining was performed by the immunoperoxidase technique after trypsinization. The antibodies used were a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:300 dilution for VEGF and a mouse monoclonal antibody (Nichirei, Tokyo, Japan) for CD34. For positive controls, tissue from a colon cancer known to express VEGF was stained for VEGF. Negative controls were done using nonspecific IgG as the primary antibody. Scoring was carried out by two independent observers blinded to the patient’s status. Positive staining was defined as the presence of VEGF immunoreactivity in at least 30% of the cancer cells.

**Evaluation of Vessel Counting.** Vessel count was assessed by light microscopy in areas of the tumor containing the highest numbers of capillaries and small venules at the invasive edge. The highly vascular areas were identified by scanning tumor sections at low power (×40 and ×100). After six areas with the highest neovascularization were identified, a vessel count was performed on a ×200 field (×20 objective and ×10 ocular), and the average counts of the six fields were determined. As Weidner et al. described (3), vessel lumens were not necessary for a structure to be defined as a vessel. The microvessels were counted by two investigators who had no knowledge of the VEGF expression of the tumors.

**Statistical Analysis.** Statistical significance was evaluated using the χ² Mann-Whitney U test. Curves for overall survival were drawn according to the Kaplan-Meier method, and differences between the curves were analyzed by applying the log-rank test. The significance level was set at 5% for each analysis.

**RESULTS**

**Expression of VEGF in Esophageal Carcinoma Cell Lines.** We initially examined VEGF mRNA expression in esophageal carcinoma cell lines. The results of a Northern blot analysis for VEGF are shown in Fig. 1. All six of the cell lines constitutively expressed 3.7-, 4.4-, and 5.5-kb VEGF mRNA at various levels. Among them, TE-2, TE-7, and TE-8 cells ex-
pressed VEGF mRNA at high levels. Southern blot analysis revealed that neither gene amplification nor rearrangement was observed in the cell lines examined (data not shown).

**Expression of VEGF in Human Esophageal Carcinoma Tissues.** Of the 119 esophageal squamous cell carcinomas, 71 (59.7%) showed intense VEGF immunoreactivity in the cytoplasm of many cancer cells (Fig. 2A). Heterogeneous staining was observed in several cases. Faint VEGF immunoreactivity was also present in some of the fibroblasts, smooth muscle cells, inflammatory cells, and vascular endothelial cells (Fig. 2B).

**Correlation between Vessel Count and VEGF Expression.** To prove that VEGF is an important angiogenic factor for human esophageal carcinoma, we performed immunohistochemistry against CD34 (endothelial cell specific), counted the microvessel number, and correlated it with VEGF expression. A representative picture is shown in Fig. 2C. The blood vessel numbers (mean ± SE) in the VEGF-negative and VEGF-positive groups were 37.6 ± 2.1 and 54.2 ± 2.9, respectively. This difference was statistically significant (Fig. 3), indicating that there was greater neovascularization in the VEGF-expressing cancers.

**Correlation between VEGF Expression and Clinicopathological Features.** We next sought to determine whether there was an association between VEGF expression, vessel count, and clinicopathological features. As shown in Table 1, VEGF positivity in the cancer cells significantly correlated with tumor stage, depth of invasion, venous invasion, and lymphatic invasion. There was no correlation between VEGF immunoreactivity and histological grade. The vessel count also correlated with the tumor stage, the depth of invasion, and lymphatic invasion (Table 1).

**Correlation between VEGF Expression and Survival Rate.** To investigate whether VEGF expression and vessel count predict the prognosis of patients with esophageal carcinoma, we selected 87 patients who were followed-up in our hospital, and Kaplan-Meier analysis was performed. Sixteen cases died of causes other than carcinoma; therefore, 71 cases were available for this analysis. At the point of this analysis, the mean follow-up time for the 25 survivors was 61.0 months (range, 25–133 months). The remaining 46 patients died between 1 and 88 months (mean, 11.2 months). The survival rate

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**Fig. 2** Immunohistochemical staining for VEGF (A and B) and CD34 (C) in human esophageal carcinoma (A and C) and normal tissues (B). Intense VEGF immunoreactivity was observed in the cytoplasm of the tumor cells (A). VEGF immunoreactivity was also detected in vascular endothelial cells (arrows), fibroblast cells (arrowhead), and smooth muscle cells (*: B). CD34 staining in the same VEGF-positive carcinoma tissues is shown (C). A and B, ×400; C, ×200.

**Fig. 3** Relationship between VEGF protein expression and microvessel counts obtained by staining for CD34. Mann-Whitney test demonstrated that $P < 0.01$. 
of patients with a high vessel count in the tumor was significantly worse than that of patients with a low vessel count in the tumor (P < 0.05). Although there was a tendency for poorer prognosis in the group with VEGF-positive tumors, this correlation was not statistically significant (Fig. 4).

**DISCUSSION**

The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by both tumor cells and normal cells (1, 2). Numerous angiogenic factors have been described; however, the ones responsible for angiogenesis in esophageal carcinoma remain unknown. VEGF is a potent stimulator of angiogenesis both in vitro and in vivo (23). Recently, we and other investigators have shown a significant correlation between VEGF expression and vessel density in several malignancies including breast (24), liver (25), pancreas (26), colon (7), and gastric carcinoma (10, 11). The importance of VEGF as a potential target for antineoplastic therapy has been demonstrated in several studies in which neutralizing antibodies to VEGF inhibited tumor growth and vascularization in vivo (27).

In the present study, the expression of VEGF was examined in esophageal carcinoma cell lines and human tumor tissues. VEGF mRNA was constitutively expressed by all of the cell lines at various levels. In the surgical specimens of esophageal carcinoma, 59.7% (71 of 119 specimens) exhibited intense VEGF immunoreactivity in cancer cells in the tumor tissues. VEGF expression was significantly correlated with neovascularization in the tumors. More interestingly, we found that the VEGF expression was significantly associated with advanced disease. Therefore, VEGF may be responsible for angiogenesis in human esophageal carcinoma.

Overexpression of VEGF has been reported in a variety of malignancies. Factors that regulate VEGF expression in tumor and nontumor cells are now being elucidated. The best-characterized mediator that induces an increase in VEGF is hypoxia (28–31). VEGF expression in cultured cells is elevated by hypoxia due to hypoxia-responsive elements that are regulated by a putative iron-containing sensor system (32). Mutation of the ras and p53 genes has been shown to up-regulate VEGF expression (33, 34). Takahashi et al. (35) have recently reported a direct correlation between p53 protein detection and VEGF expression in human colon cancer. Although allelic loss and mutation of the p53 gene are common events of esophageal carcinoma occurring from an early stage (36), our preliminary study revealed no correlation between VEGF expression and accumulation of p53 protein in esophageal carcinoma cells.

Numerous cytokines and growth factors produced by tumor and normal cells affect VEGF expression. It has been reported that VEGF expression is enhanced by epidermal growth factor (37), platelet-derived growth factor BB (37), transforming growth factor β (38), insulin-like growth factor I (39), insulin-like growth factor II (40), IL-1α (11), and IL-6 (41). Because esophageal carcinoma frequently overexpress epidermal growth factor and its receptor (42, 43), it might result in the constitutive overexpression of VEGF in tumor cells via an autocrine mechanism.

Univariate analysis of the immunohistochemical data demonstrated that the presence of VEGF in the esophageal carcinoma cells is associated with enhanced tumor extension and greater neovascularization, indicating that VEGF has the potential to contribute to tumor growth in vivo. These data are consistent with the findings of Inoue et al. (44). In this study, lymphatic invasion was also associated with VEGF positivity and greater vessel counts. Although angiogenesis refers to an increase in blood vessel formation, others have found that angiogenesis also corresponds to increased lymph node metastasis (45). It has been demonstrated that neovascularization of the rabbit cornea after the injection of India ink leads to the appearance of ink particles in the ipsilateral lymph nodes (46). These
findings indicate that lymphocapillary anastomoses are present and/or that angiogenesis correlates with the formation of new lymphatic vessels.

The prognosis and choice of therapy for most esophageal carcinoma patients are based on both the histological type and the tumor stage. Previous studies have demonstrated that angiogenesis is associated with the prognosis of patients with several malignancies (3–8, 47). We followed patients to determine whether a higher vessel count and VEGF expression can predict which patients are likely to have recurrences. As we expected, the prognosis of patients was significantly associated with the vessel count. Although there was a tendency for shorter survival in the VEGF-positive group, this correlation did not reach statistical significance, because tumor angiogenesis is not simply controlled by the presence of VEGF but may be mediated by several angiogenic factors. Because the angiogenic process is complex, additional studies concerning other angiogenic regulators should be performed.

In conclusion, this study has demonstrated that VEGF expression is associated with advanced disease as well as vessel density in human esophageal carcinoma. The identification of factors that correlate with angiogenesis in esophageal carcinoma may provide a basis for experiments targeting these angiogenic factors to inhibit the vascularization of tumors. It is possible that VEGF may be used as a target for antiangiogenic therapy in the future.

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