Significance of Vessel Count and Vascular Endothelial Growth Factor and Its Receptor (KDR) in Intestinal-type Gastric Cancer

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ABSTRACT

Angiogenesis is essential for tumor growth and metastasis and depends on the production of angiogenic factors by host and tumor cells. The role of angiogenesis and angiogenic factor expression in intestinal- and diffuse-type gastric cancer are undefined. Archival specimens of 51 intestinal-type and 38 diffuse-type human gastric carcinomas were examined for tumor vessel counts, angiogenic factor expression, and the presence or absence of angiogenic factor receptors on tumor endothelium using antibodies against vascular endothelial growth factor (VEGF) and its receptors (KDR and flt-1), basic fibroblast growth factor (bFGF) and its receptors (bek and flg), and factor VIII (endothelial cells). Vessel count and VEGF and bFGF expression were higher in intestinal-type than in diffuse-type gastric cancers (P = 0.01, P < 0.001, and P < 0.001, respectively). Similarly, vessel count and VEGF expression were higher in patients with liver metastasis than in patients with peritoneal dissemination (P = 0.003 and P = 0.01, respectively). Vessel count correlated with VEGF expression and the presence of endothelial KDR in intestinal-type gastric cancer (P = 0.003 and P = 0.02, respectively) but not diffuse-type gastric cancer. Vessel count, VEGF expression, and presence of endothelial KDR increased with increasing stage of disease in intestinal-type gastric cancer but not diffuse-type gastric cancer. The expression of bFGF and its receptors did not correlate with vessel count in either cancer type. These findings suggest that the pattern of metastasis in intestinal-type gastric cancer is angiogenesis dependent. The correlation of VEGF expression and its endothelial receptor with vessel count and stage of disease suggests that VEGF is at least one of the factors responsible for the induction of angiogenesis in intestinal-type gastric cancer.

INTRODUCTION

Gastric cancer prognosis is dependent on both pathological tumor type and stage of disease (1, 2). The intestinal type of gastric cancer tends to be exophytic, metastasizing to the liver by hematogenous dissemination. In contrast, the diffuse type of gastric cancer is more invasive, with predominantly peritoneal dissemination. The factors responsible for liver metastasis and peritoneal dissemination have not yet been identified.

Angiogenesis is essential for tumor growth and metastasis and depends on the production of angiogenic factors by host and/or tumor cells (3). Increased vascularity may allow not only an increase in tumor growth but also a greater chance for hematogenous metastasis (4). Weidner et al. (5) showed a correlation between the incidence of metastasis and microvessel count in invasive breast carcinomas. Similar studies have confirmed this finding in other malignancies, including lung cancer (6), prostate cancer (7), melanoma (8), cervical cancer (9), and colon cancer (10).

Angiogenesis is not a passive process and is driven by the production of tumor and host-derived angiogenic factors. Of the known angiogenic factors, two well-characterized peptides, VEGF (11, 12) and bFGF (13, 14), have been shown to induce angiogenesis in rodent tumor models. The receptors for these factors have been characterized and demonstrated on tumor endothelium (15–18). Previous studies from our laboratory have demonstrated the importance of these factors in human colon cancer metastasis (10, 19).

Because of the unique growth characteristics and metastatic patterns of the intestinal and diffuse types of gastric cancers, we hypothesized that the intestinal type of gastric cancer is more angiogenesis dependent than the diffuse type of gastric cancer. Therefore, the purpose of this study was to determine whether there are differences in vessel count and expression of VEGF and bFGF between the intestinal and diffuse types of gastric cancers. We also sought to determine whether specific angiogenic factors are associated with vessel count and if the receptors for these factors are simultaneously present on endothelial cells.

PATIENTS AND METHODS

Patients and Tumor Specimens. Paraffin-embedded tumor specimens from 89 randomly selected patients with gastric carcinomas who had undergone surgery at the Cancer Research Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-6926; Fax: (713) 792-0722.
growth factor (>50% homology) and platelet-derived growth factor (~20% homology). By Western blot analysis, the antibody for VEGF detected only VEGF, and there was no cross-reactivity with placenta growth factor or platelet-derived growth factor (data not shown).

For positive controls, tissue from a colon cancer known to express VEGF was stained for VEGF, tissue from a bladder tumor was stained for bFGF, umbilical vein tissue was stained for KDR and flt-1, and normal liver tissue was stained for bek and flg. Negative controls were done using nonspecific IgG as the primary antibody.

Evaluation of Immunostaining and Vessel Counting. The intensity of staining for VEGF and bFGF was evaluated blindly at the invasive edge and was confirmed by an image analyzer using the Optimas software program (Bioscan, Edmonds, WA). Intensity of staining for VEGF and bFGF was graded on a scale of 0 to 3+, with 0 representing no detectable stain and 3+ representing the strongest stain (Fig. 1). The presence or absence of KDR, flt-1, bek, and flg was evaluated on both tumor endothelial cells and tumor epithelial cells.

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 the area of highest neovascularization was identified, a vessel count was performed on a ×200 field (×20 objective and ×10 ocular, 0.739 mm² per field). As Weidner et al. described (5), vessel lumens were not necessary for a structure to be defined as a vessel.

Statistical Analysis. Differences in vessel count and in mean intensity of VEGF and bFGF staining between intestinal-type tumors and diffuse-type tumors were analyzed by Student’s t test. Differences in mean vessel count between receptor-positive and receptor-negative tumors were also analyzed by Student’s t test. Correlations between stage of disease and vessel count, expression of VEGF and bFGF, and vessel count, and intensity of VEGF and bFGF staining were examined by the Spearman rank correlation coefficient. Differences in rates of positivity for the receptors among subgroups were assessed by χ² analysis. All statistical analyses were performed using Statworks statistical software (Cricket Software, Inc., Philadelphia, PA), and differences were deemed significant at the 95% confidence interval.

RESULTS

Vessel Count and VEGF and bFGF Expression in Tumors. The intensity of VEGF and bFGF staining was homogeneous within tumors, and there were no detectable “hot spots.” Vessel count, VEGF expression, and bFGF expression were all significantly higher in intestinal-type tumors than in diffuse-type tumors (P = 0.01, P < 0.001, and P < 0.001, respectively; Table 2).

Vessel count (P = 0.003) and VEGF expression (P = 0.01) were significantly higher in patients with liver metastasis than in patients with peritoneal dissemination. bFGF expression did not differ between the two groups of patients. Photomicrographs of representative intestinal-type and diffuse-type gastric cancers stained for factor VIII, VEGF, and bFGF are shown in Fig. 2.

### Table 1. Clinical characteristics of gastric cancer patients

<table>
<thead>
<tr>
<th>Stage of disease</th>
<th>Intestinal type (n = 51)</th>
<th>Diffuse type (n = 38)</th>
<th>Total (n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean), yr</td>
<td>39–79 (59.2)</td>
<td>33–68 (53.5)</td>
<td>33–79 (59.6)</td>
</tr>
<tr>
<td>Male/female</td>
<td>36/15</td>
<td>19/19</td>
<td>55/34</td>
</tr>
</tbody>
</table>

* Stage 1: T1, N0, M0; or T2, N0, M0. Stage 2: T1, N0, M0; T2, N1, M0, or T1, N1, M0. Stage 3: T2, N0, M0; T3, N1, M0; or T1, N0, M1, M0. Stage 4: T3, N0, M1; or T4, N0, M0, T1 tumor confined to lamina propria, submucosa. T2 tumor penetrates serosa. T3 tumor invades adjacent structures. N1, positive perigastric lymph node <3 cm from primary. N2, positive lymph node >3 cm from primary, along left gastric, common hepatic, splenic, or celiac arteries. M0, no distant metastasis. M1, distant metastasis.
Fig. 1 Subjective evaluation of staining intensity for VEGF in tumor cells at the invasive edge was done blindly. To confirm the validity of this assessment, five representative specimens from each group were selected. Integrated absorbance of 12 distinct areas (5 μm in diameter, indicated by circles) within the tumor cell cytoplasm were obtained (A). The average intensity was found to correlate closely with the subjective findings, in that tumors with a higher subjective score demonstrated a higher intensity by image analysis. In B, representative staining for VEGF is demonstrated for each grade.

Table 2 Vessel count and staining intensity in primary tumors from patients with gastric cancer, liver metastases, and peritoneal dissemination

<table>
<thead>
<tr>
<th>Histological and metastatic types</th>
<th>Vessel count (mean ± SE)</th>
<th>Staining intensity (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td>Intestinal type</td>
<td>37.2 ± 3.4</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>26.1 ± 2.5</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>(n = 51)</td>
<td></td>
<td>P=0.01</td>
</tr>
<tr>
<td>(n = 38)</td>
<td></td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Primary gastric cancer with liver metastasis</td>
<td>54.9 ± 5.0</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Primary gastric cancer with peritoneal dissemination</td>
<td>26.8 ± 3.9</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>(n = 15)</td>
<td></td>
<td>P=0.003</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td>P=0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bFGF</td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.1</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Correlations between Stage of Disease and Vessel Count, VEGF Expression, and bFGF Expression. As shown in Fig. 3, vessel count correlated with stage of disease in patients with intestinal-type tumors. Similarly, the intensity of VEGF staining also correlated with stage of disease in patients with intestinal-type tumors. In patients with diffuse-type gastric tumors, there were no correlations among vessel count, VEGF expression, and stage of disease. The intensity of bFGF staining did not correlate with stage of disease in either tumor type.

Correlations between Vessel Count and VEGF and bFGF Expression. A correlation between tumor vessel count and VEGF expression was observed in specimens from patients with intestinal-type tumors (P = 0.003) but not in specimens from patients with diffuse-type tumors. There was no correlation between vessel count and bFGF expression in either tumor type.

Positivity for KDR, flt-1, bek, and fig on Tumor Endothelia and Tumor Epithelial Cells. As shown in Table 3, positivity for KDR on tumor endothelium occurred significantly more often (39.2%) in intestinal-type tumors than in diffuse-type tumors (15.8%; P = 0.02). Seventeen (77.3%) of 22 intestinal-type tumors positive for KDR on endothelium were from patients with stage 3 or 4 disease. There were no differences in positivity for the other VEGF and bFGF receptors (flt-1, bek, and fig) on tumor endothelium in either intestinal-type or diffuse-type gastric tumors.

KDR was not detected on tumor cells, and <10% had minimal staining for flt-1. In contrast, the receptors for bFGF (bek and fig) were detected on tumor cells in 83.5 and 77.8%, respectively, of the tumors studied. Expression of bFGF on tumor cells correlated with the presence of bek and fig on tumor cells (P = 0.004 and P = 0.003, respectively).

Correlations between Vessel Count and Receptor Positivity. The mean vessel count in intestinal-type gastric tumors in which the endothelium stained positive for KDR was significantly higher than in those in which the endothelium stained negative (P = 0.02); however, there was no difference in vessel count between diffuse-type tumors with KDR-positive- or KDR-
negative-staining tumor endothelium. Furthermore, there was no difference in vessel count in either diffuse-type or intestinal-type tumors in patients with flt-1-, bek-, or flg-positive or -negative staining tumor endothelium.

DISCUSSION

Both the growth patterns and the biological behavior of intestinal-type and diffuse-type gastric carcinomas are distinct. Intestinal-type gastric cancers demonstrate exophytic growth
and commonly metastasize to the liver, whereas diffuse-type tumors demonstrate invasive growth and peritoneal metastasis (1, 2). The Lauren classification of gastric cancer is based on the pathobiology of cell cohesion (20, 22). In intestinal-type gastric cancer, the neoplastic cells adhere to each other, forming structures that resemble intestinal mucosa. In contrast, cells of diffuse-type gastric cancer lack these adhesive qualities and infiltrate the gastric wall either as individual cells or in nests (22). Those countries with a high rate of gastric cancer (such as Japan) have a high percentage of patients who present with the intestinal-type of disease (22). In our study of Japanese patients with gastric cancer, nearly 60% presented with the intestinal type. In other areas of the world, the distribution of this disease is relatively equally divided between intestinal- and diffuse-type gastric cancer. Despite the relatively distinct microscopic characteristics and growth patterns of the two main types of gastric cancer, the overall survival for both patient populations is similar (35–45%; Ref. 22). Although some believe that the specific types of gastric cancer may evolve from one type to another (intestinal to diffuse), we do not believe this to be the case. Poorly differentiated intestinal-type carcinoma has solid (i.e., nongland-forming) areas, but this is not equivalent to evolution to the diffuse-type cancer. Occasionally, tumors may be mixed with components of both intestinal and diffuse types. Our study excluded the mixed type of gastric cancer from analysis (~5% of gastric cancers).

We hypothesized that angiogenesis and its regulating factors play a role in determining the growth characteristics and patterns of metastasis in diffuse-type and intestinal-type gastric cancers. We found that vessel count, VEGF expression, and bFGF expression were significantly higher in intestinal-type gastric tumors than in diffuse-type tumors. Significant correlations among stage of disease, vessel count, and VEGF expression were observed in intestinal-type tumors. Moreover, tumors associated with liver metastasis (all were intestinal-type tumors) showed higher vessel counts and VEGF expression than tumors associated with peritoneal dissemination. These results suggest that the processes of growth and metastasis in intestinal-type tumors are more angiogenesis dependent than they are in diffuse-type tumors. Furthermore, the correlation of VEGF expression and vessel count implies that VEGF may induce the angiogenic response in intestinal-type gastric cancer. These data strongly suggest that VEGF may be the more important of the two angiogenic factors studied in inducing neovascularization in intestinal-type gastric tumors.

Expression of bFGF also was higher in intestinal-type gastric tumors than in diffuse-type gastric tumors, but bFGF expression did not correlate with vessel count or with stage of disease. bFGF is a ubiquitous, potent growth factor that can act as a tumor cell mitogen as well as an angiogenic factor. This study demonstrated the presence of bFGF receptors on gastric tumor cells, which suggests that bFGF acts as a tumor cell mitogen for gastric carcinoma cells in an autocrine and/or paracrine fashion. This theory is supported further by the fact that tumor cells with high bFGF expression were more likely to stain positive for bFGF receptors than tumor cells with lower bFGF expression. Thus, it is possible that bFGF acts as a tumor cell mitogen and VEGF acts as an angiogenic factor in intestinal-type gastric cancer.

To confirm the hypothesis that VEGF plays a role in angiogenesis in intestinal-type gastric cancer, it must be demonstrated that the receptors for this factor are present on tumor endothelium. The significance of expression of VEGF and its receptors has been reported in other human carcinomas, such as

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**Table 3**  Tumor endothelium positivity for receptors for VEGF and bFGF in gastric cancer patients

<table>
<thead>
<tr>
<th>Type of gastric tumor</th>
<th>Receptors for VEGF</th>
<th>Receptors for bFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal type</td>
<td>KDR</td>
<td>flt-1</td>
</tr>
<tr>
<td>(n = 51)</td>
<td>20° (39.2%)</td>
<td>10° (19.6%)</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>6° (15.8%)</td>
<td>5° (13.2%)</td>
</tr>
</tbody>
</table>

*p = 0.02

*Number of tumors where receptors are present on tumor endothelium.
colon cancer (15), breast cancer (17), and brain tumors (18). In a small series of patients, Brown et al. (15) demonstrated the presence of VEGF and its receptors in gastric cancer specimens. To date, no study has examined the importance of angiogenic factors, neovascularization, and the clinical course of patients with specific types of gastric cancer. In our study, a greater percentage of intestinal-type gastric tumors than diffuse-type gastric tumors expressed KDR on tumor endothelium (39.2% versus 15.8%; P = 0.02). Most KDR-positive tumors were from patients with advanced stages of disease. Moreover, intestinal-type tumors in which the endothelium stained positive for KDR were associated with higher vessel counts than were KDR-negative tumors. There were no differences in endothelial positivity for the other receptors (flt-1, flk-1, and flg) between intestinal-type and diffuse-type tumors. These results suggest that VEGF and KDR may be the important receptor-ligand system in the process of angiogenesis in intestinal-type gastric cancer. If VEGF is indeed responsible for tumor angiogenesis in intestinal-type gastric cancer, strategies using antibodies or antisense RNA to VEGF may inhibit tumor angiogenesis. To address this hypothesis, we plan to down-regulate VEGF expression in tumor cells using an orthotopic implantation model of gastric cancer. The different growth characteristics and metastatic patterns of intestinal-type and diffuse-type gastric cancers may be due, in part, to the increased vascularity of intestinal-type tumors. This may be caused by tumor secretion of VEGF and binding to its receptor on tumor endothelium. The correlation of vessel count, VEGF expression, and stage of disease in intestinal-type gastric cancer suggests that angiogenesis may be a potential prognostic marker in this type of gastric cancer. In addition, VEGF may provide a potential target for therapy in intestinal-type gastric cancer. ACKNOWLEDGMENTS

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