Significance of Platelet-derived Endothelial Cell Growth Factor in the Angiogenesis of Human Gastric Cancer

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ABSTRACT

We have previously shown that platelet-derived endothelial cell growth factor (PD-ECGF) is associated with angiogenesis of human colon cancer; this factor is expressed at high levels in vascular tumors that express low levels of vascular endothelial growth factor (VEGF). In these colon cancers, the major source of PD-ECGF is the infiltrating cells. In this study, we examined the role of PD-ECGF in the angiogenesis of human gastric cancer. Immunostaining for PD-ECGF was done on 93 gastric cancers previously stained for VEGF, basic fibroblast growth factor, and factor VIII-related antigen (specific for endothelial cells). To determine the cell type expressing PD-ECGF, double staining was done using antibodies to both PD-ECGF and CD68 (specific for macrophages). PD-ECGF was expressed more frequently in infiltrating cells (positive CD68 staining; 53.8%) than in tumor epithelium (9.7%; P < 0.0001). Infiltrating cells simultaneously stained positive for both PD-ECGF and CD68. An association between PD-ECGF expression in infiltrating cells, VEGF expression in tumor epithelium, and vessel count was observed in intestinal-type gastric cancer but not in diffuse-type gastric cancer. Vessel count was greater in tumors with high expression of both PD-ECGF and VEGF than in those with high expression of either factor alone (P = 0.002). Multiple angiogenic factors expressed by both tumor cells and infiltrating cells may play a role in the regulation of angiogenesis in intestinal-type gastric cancer.

INTRODUCTION

Angiogenesis is essential for tumor growth and metastasis and depends on the production of angiogenic factors by tumor cells and/or infiltrating cells (1, 2). Increased vascular density is associated with a high incidence of metastases in many tumors including breast (3), skin (4), cervical (5), prostate (6), colon (7, 8), and other cancers. We have previously shown that VEGF3 is associated with the angiogenesis of colon cancer (7, 8) and intestinal-type gastric cancer (9). However, it is unlikely that a single angiogenic factor regulates the angiogenic process in any tumor system.

PD-ECGF, an endothelial cell mitogen initially purified to homogeneity from human platelets (10, 11), has chemotactic activity for endothelial cells in vitro and is angiogenic in vivo (11). PD-ECGF has also been demonstrated to be identical to thymidine phosphorylase, an enzyme involved in pyrimidine nucleoside metabolism (12). PD-ECGF expression is elevated in several solid tumor types (13–18). We have recently demonstrated that PD-ECGF expression in human colon cancer specimens is associated with vessel count; double staining techniques showed that the cells of origin of PD-ECGF in colon cancers are the infiltrating cells (19).

The biological behavior of gastric cancer depends on the pathological tumor type (20, 21). Intestinal-type tumors tend to be exophytic, metastasizing to the liver by hematogenous dissemination. In contrast, diffuse-type gastric cancers are more invasive, with predominantly peritoneal dissemination. Although the factors responsible for liver metastasis and/or peritoneal dissemination have not yet been identified, we have previously shown that intestinal-type gastric cancer is more angiogenic than the diffuse type (9).

In this study, we investigated whether an association exists between PD-ECGF expression and angiogenesis in gastric cancer. We also sought to determine whether PD-ECGF expression correlates with the expression of other angiogenic factors, and whether PD-ECGF expression differs in intestinal-type and diffuse-type gastric cancers.

MATERIALS AND METHODS

Patients and Tumor Specimens. Paraffin-embedded tumor specimens from 93 randomly selected patients with gastric cancers who had undergone surgery at the Cancer Research Institute, Kanazawa University (Kanazawa, Japan) were studied. The pathology reports and clinical histories were reviewed to determine the correct disease stage. A staff pathologist at the University of Texas M. D. Anderson Cancer Center (K. R. C.) reevaluated the tumors for pathological subtype. Using the Lauren classification system, the specimens studied were divided into intestinal-type (50 patients) and diffuse-type (43 patients)

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Table 1  Clinical characteristics of gastric cancer patients

<table>
<thead>
<tr>
<th>Intestinal type</th>
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<th>Total</th>
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<tr>
<td>(n = 50)</td>
<td>(n = 43)</td>
<td>(n = 93)</td>
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<tr>
<td>Age (mean yr)</td>
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<td>35-68 (53.8)</td>
</tr>
<tr>
<td>Male-female</td>
<td>36:14</td>
<td>21:22</td>
</tr>
<tr>
<td>Stage of diseasea</td>
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<td>2</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
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</tr>
<tr>
<td>4</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

* Stage 1, T1,N0,M0 or T1,Nx,Mx. Stage 2, T2,Nx,Mx or T2,N1,M0. Stage 3, T3,Nx,Mx or T3,N1,Mo. Stage 4, T4,Nx,M0 or T4,Nx,Mx. T1, tumor confined to lamina propria or submucosa. T2, tumor confined to muscularis propria or subserosa. T3, tumor penetrates serosa. T4, tumor invades adjacent structures. N0, no regional lymph node metastasis. N1+, regional lymph node metastasis. M0, no distant metastasis. M1, distant metastasis.

Evaluation of Immunostaining and Vessel Counting. The intensity of staining for VEGF, bFGF, and PD-ECGF at the invasive edge in tumor cells was graded on a scale of 0–3+, with 0 representing no detectable stain, and 3+ representing the strongest stain (9). Intensity of staining of PD-ECGF-positive infiltrating cells was similarly on a scale of 0–3+, as follows: 0, no PD-ECGF-positive infiltrating cells; 1+, slight staining intensity; 2+, moderate staining intensity; 3+, strongest staining intensity of PD-ECGF in infiltrating cells. To confirm our evaluation, image analysis was done on representative specimens from each gradation of staining using the Optimas software program (Bioscan, Edmonds, WA) as described previously (9).

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, based on the criteria of Weidner et al. (3). The highly vascular areas were identified by scanning tumor sections at low power (×40 and ×100). After the area of highest neovascularization was identified, individual vessels were counted in a ×200 field (×20 objective and ×10 ocular; 0.739 mm²/field). These evaluations were done without knowledge of the patient’s clinical status.

Statistical Analyses. Correlations among angiogenic factor expression, stage, and vessel count were determined by the Spearman rank correlation coefficient. Differences in PD-ECGF expression among the stages were assessed by Student’s t test, and differences in the rate of positive PD-ECGF staining were assessed by χ² analysis. All statistical differences were deemed significant at the 95% confidence interval. All analyses were done using InStat 2.01 statistical software (GraphPad Software, San Diego, CA).

RESULTS

Immunohistochemical Staining for PD-ECGF. Of the 93 patient samples examined, 50 (53.8%) demonstrated PD-ECGF protein in infiltrating cells (Table 2). In contrast, only nine tumors (9.7%) stained positive for PD-ECGF in tumor epithelium (P < 0.0001, χ² analysis). We did not detect significant heterogeneity in staining intensity. PD-ECGF expression in infiltrating cells was observed in 33 intestinal-type gastric cancers (66.0%) but only 17 (39.5%) of 43 diffuse-type gastric cancers; this difference was significant (P = 0.019, χ² analysis).

The majority of PD-ECGF-positive cells also stained positive for CD68 (specific for macrophages; Fig. 1).

Correlation between PD-ECGF Expression, Tumor Stage, Vessel Count, VEGF Expression, and bFGF Expression in Intestinal-Type Gastric Cancer. PD-ECGF expression in infiltrating cells correlated with vessel count in intestinal-type gastric cancer (Spearman r = 0.3846; P = 0.0070) but not in diffuse-type gastric cancer. PD-ECGF expression in infiltrating cells in intestinal-type gastric cancer also correlated with VEGF expression (Spearman r = 0.4987; P = 0.0003), but there was no such correlation in diffuse-type gastric cancer. Expression of PD-ECGF in infiltrating cells did not differ by stage of disease in either intestinal- or diffuse-type gastric cancer. There was no correlation between PD-ECGF expression and bFGF expression in either intestinal- or diffuse-type gastric cancer.

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Vessel Count in Tumors with High Expression of Both PD-ECGF and VEGF. We hypothesized that tumors with high expression of both VEGF and PD-ECGF would have higher vessel counts than tumors with high expression of either factor alone. We therefore compared the vessel counts in tumors in which VEGF expression and PD-ECGF were both ≥2+ to those in which VEGF expression or PD-ECGF expression was ≤1+. The mean vessel count was 54.5 ± 7.7 (mean ± SE) in the former group, which was significantly higher than the mean of 31.4 ± 3.1 in latter group (P = 0.002).

DISCUSSION

The process of angiogenesis is the outcome of an imbalance between positive and negative regulators of neovascularization. Since Folkman’s initial discovery that tumors are angiogenesis dependent (1, 2), a myriad of positive and negative regulators of angiogenesis have been discovered. Although many factors may induce angiogenesis in vivo (9), not all induce endothelial cell proliferation in vitro. Thus, it is possible that some angiogenic factors act through a second messenger system either by inducing the expression of other angiogenic factors or by contributing to angiogenesis through some type of enzymatic activity.

PD-ECGF has been demonstrated to induce chemotaxis of endothelial cells and angiogenesis in vivo, but it does not stimulate the growth of endothelial cells in vitro (9). Its amino acid sequence does not contain a signal peptide; therefore, PD-ECGF is not a classic paracrine growth factor. In fact, the amino acid sequence for PD-ECGF has been found to be identical to that of thymidine phosphorylase (24), whose enzymatic activity is necessary for angiogenesis (25).

Because of the complex nature of the angiogenic process, it is unlikely that any single factor is responsible for angiogenesis in a particular tumor type. In each individual tumor, there may be a dominant angiogenic factor that causes an imbalance of the positive and negative regulators to induce angiogenesis. In previous studies from our laboratory, we noted a correlation between VEGF expression and vessel count in human colon cancers (7). However, we also noted that several tumors exhibited a very high vessel count but had relatively low levels of VEGF. To determine whether another angiogenic factor was responsible for this increase in angiogenic activity, we investigated the role of PD-ECGF in human colon cancer angiogenesis. Using immunohistochemical techniques, we found that the colon cancer epithelium did not stain for this factor; instead, the majority of staining was in the infiltrating cells. We also found that PD-ECGF expression in the infiltrating cells in human colon cancer specimens correlated with vessel count.

In the present study, our findings in intestinal-type gastric cancer were similar to those in our previous studies (19), i.e., we found that PD-ECGF expression in infiltrating cells is associated with vessel count. Northern blot analysis demonstrated that gastric cancer cell lines (a pure population of gastric cancer epithelium) express less PD-ECGF than do gastric mucosa or gastric cancer specimens (data not shown). This provides indirect evidence that infiltrating cells are the predominant source of PD-ECGF, although the gastric cancer epithelium also expresses PD-ECGF, albeit at a lower level. In PD-ECGF-driven angiogenesis, the host immune cells may be the driving force behind this neovascularization. Immune cells are known to express other angiogenic factors, including bFGF (26), transforming growth factor α (27), platelet-derived growth factor (28), VEGF (29), and interleukin 8 (15). The observation that infiltrating cells may contribute to angiogenesis in solid malignancies was initially made by Polverini and Leibovich (30) and Leibovich et al. (31) in both in vivo and in vitro systems. Those authors demonstrated that tumor-associated macrophages and their conditioned media induce neovascularization in the cornea. Their studies also suggested that tumor-associated macrophages induce a stronger angiogenic response than do peritoneal macrophages, and the authors found that conditioned media from tumor-associated macrophages induce a 10-fold increase in endothelial cell proliferation.

By using the double staining technique, we were able to demonstrate that PD-ECGF is expressed predominantly by infiltrating cells in intestinal-type gastric cancers. We noted very weak staining of the tumor epithelium itself, consistent with our findings in colon cancer. Similar findings were noted by Takebayashi et al. (32) in a study of colon cancers and adenomas. These investigators found increased PD-ECGF expression in colon cancer compared to that in adenomas or normal mucosa (32). In contrast, studies in breast cancers demonstrated staining for PD-ECGF in the tumor epithelium without notation to the presence or absence of PD-ECGF in infiltrating cells (18, 33). However, similar to our studies, the studies in breast cancer specimens demonstrated a correlation between PD-ECGF expression and vessel count.

The effect of PD-ECGF on angiogenesis may not be direct but may be through another angiogenic factor or through facilitation of endothelial cell invasion by its enzymatic activity. It is interesting to note that the highest vessel counts were observed in those tumors with high expression of both VEGF and PD-ECGF. Toi et al. (18) observed similar findings in primary breast cancers. In that series, tumors with the highest vessel counts expressed both VEGF and PD-ECGF in 75.5% of cases. It is possible that the activity of one factor facilitates the angio-

<table>
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<th>Tumor type</th>
<th>No. positive (%)</th>
<th>Intensity</th>
<th></th>
<th>No. positive (%)</th>
<th>Intensity</th>
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<td></td>
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<td>18 11 4</td>
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<tr>
<td>Diffuse type</td>
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<td>1+ 2+ 3+</td>
<td></td>
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<td>6 10 1</td>
</tr>
<tr>
<td>Total (n = 93)</td>
<td>9 (9.7)</td>
<td>1+ 2+ 3+</td>
<td></td>
<td>50 (53.8)</td>
<td>24 21 5</td>
</tr>
</tbody>
</table>

Table 2  Expression of PD-ECGF protein in gastric cancer epithelium and infiltrating cells

Clinical Cancer Research
Fig. 1 Immunohistochemical staining of gastric cancer specimens using antibodies to PD-ECGF (A) or PD-ECGF and CD68 (B); ×440. A, PD-ECGF protein is observed in the infiltrating cells, but not in tumor epithelial cells. B, double staining demonstrates that infiltrating cells stain for both PD-ECGF and CD68 (arrows).

genic activity of the other factor. For example, the chemotactic activity of PD-ECGF may facilitate tubule formation after proliferation of endothelial cells secondary to VEGF activity. Alternatively, the same conditions may induce high expression of both of these angiogenic proteins, inducing a strong angiogenic response. For example, in separate investigations, it has been demonstrated that hypoxia induces expression of both VEGF and PD-ECGF (34–36). Similarly, it is possible that specific cytokines and/or growth factors may induce both VEGF and PD-ECGF (37, 38). Whether PD-ECGF and VEGF are redundant, additive, or synergistic remains to be determined.

In our studies, the association of PD-ECGF with angiogenesis was observed only in intestinal-type gastric cancer. 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 (20, 21).
Lauren classification of gastric cancer is based on the pathobiology of cell cohesion (22, 39). 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 (39). Countries with a high rate of gastric cancer (such as Japan) have a high percentage of patients who present with the intestinal type of this cancer (39). In our study of Japanese patients with gastric cancer, more than half presented with the intestinal type (9). Approximately 5% of gastric cancers have components of both intestinal and diffuse types. Our study excluded this mixed type of 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. 39). Although some believe that 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., non-gland-forming) areas, but this is not equivalent to evolution to diffuse-type cancer.

In summary, high vessel count is associated with high PD-ECGFR expression in intestinal-type gastric cancer, and the major source of PD-ECGF seems to be the infiltrating cells. PD-ECGFR expression is also associated with VEGF expression in the tumor epithelium. In contrast, there were no associations among these factors in diffuse-type gastric cancer. In intestinal-type gastric cancer, PD-ECGF and VEGF may be additive or synergistic in their action, because high expression of both angiogenic factors leads to the highest vessel count. Understanding mechanisms of gastric cancer angiogenesis and the angiogenic phenotype provides a basis for a rational approach to the development of antiangiogenic therapies in patients with intestinal-type gastric cancer (40).

REFERENCES


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