Association of Placenta Growth Factor and Vascular Endothelial Growth Factor with Angiogenesis and Clinical Outcome in Human Gastric Cancer

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Introduction

Angiogenesis is a very complex phenomenon and essential for the growth of solid tumors measuring more than a few millimeters. It permits rapid tumor growth and potential presence of tumor metastasis. Tumor angiogenesisis is a significant predictor of prognosis and hematogenous metastasis of patients with gastric cancer.

Angiogenesis is not a passive process and is driven by many angiogenic factors produced by tumor. Of the known angiogenic factors, vascular endothelial growth factor (VEGF) have been shown to play an important role not only in angiogenesis and prognosis of different human tumors, but also in physiological and nonmalignant pathological conditions.

Placenta growth factor (PlGF) is a member of the VEGF family of proangiogenic factors. VEGF binds with high affinity to two tyrosine kinase receptors: VEGF receptor-1 (Flt-1) and VEGFR-2 (Flk-1). PlGF binds with high affinity to Flt-1 but not to Flk-1. In contrast to VEGF, PlGF is not regulated by hypoxia and its physiological roles are largely unknown. PlGF is upregulated in placenta and during the active angiogenic phase of wound healing. Its upregulation has been found in human meningiomas, hemangioblastomas, melanoma, cervical squamous cell carcinoma and associated with angiogenesis in renal cell carcinoma. However, PlGF was down regulated in thyroid carcinoma, germ cell tumor, and cervical

adenocarcinoma. To our knowledge, there has been no report on PlGF expression of human gastric cancer and its correlation with clinical outcome.

Therefore, we quantitated expression of VEGF and PlGF in human gastric cancer tissues, which represent an important mechanism of angiogenesis, by enzyme immunoassays and compared with microvessel density and other clinicopathological variables as a basis for rational future therapeutic antiangiogenic approaches to gastric cancer treatment.

Materials and Methods

Patients. A total of 79 patients with gastric cancer, who had undergone radical gastrectomy at our institution from July 1995 to March 1999, were included in this study. They were all proved to have adenocarcinomas by panendoscopic biopsies. They were staged according to TNM system. Criteria for consideration as curative resection were the complete removal of a primary gastric tumor, D2 dissection of regional lymph nodes, and no macroscopic tumor being left behind. They had no detectable metastasis in liver, peritoneum and distant organ at the time of surgery. No other previous or concomitant primary cancer was present. No patient had received chemotherapy and radiotherapy before surgery. Clinicopathologic factors including age, sex, gross types of tumors (Borrmann classification), histologic types of tumors (Lauren classification), depth of tumor invasion, and status of lymph node metastasis documented with histologic findings were reviewed and stored in patients' database. The patients were followed up from 3 to 46 months after sugery. The follow-up intervals were calculated as survival intervals after surgery.

Microvessel Staining and Evaluation. The paraffinized tumor blocks of 79 patients whose gastric cancers were stained for endothelial cell CD34 antigen using the labelled streptavidin-biotin after antigen retrieval (Fig. 2). Briefly, deparaffinized sections were heated in a pressure cooker. After endogenous peroxidase was blocked

with 3% hydrogen peroxide in the section, each section was incubated with nonimmuned horse serum. The sections were incubated in anti-CD34 monoclonal antibody (Santa Crus, CA) at a dilution of 1:20, or the control nonimmune serum at 4 ℃ overnight. The sections were incubated with link antibodies followed by peroxidase conjugated streptavidin complex (LSAB kit, DAKO Corporation, Carpinteria, CA). The peroxidase activity was visualized with diaminobenzidine tetrahydroxychioride solution (DAB, DAKO corporation, Carpinteria, CA) as the substrate. The sections were lightly counterstained with hematoxylin. After screening the areas with intense neovascularized spots at low power field (100X), microvessels in the area with the highest number of discrete microvessels were counted in a 400X field. Three separate intense neovascularized areas were assessed, and the mean was calculated as microvessel density of each tumor evaluated.

Extraction of the Tumor Cytosols. Protein lysate from each specimen was prepared using 10 mg tissue cut into tiny pieces, suspended in cell lysis buffer (0.15 M NaCl; 0.1 M Tris [pH 8.0]; 1 mM EDTA [pH 8.0]; 1 mM PMSF) and mechanically homogenized with a polytron PT 3000 (30,000 r.p.m. for one minute).

Quantification of PlGF and VEGF levels in cancer and non-tumor tissue of the same resected stomach.

Concentrations of VEGF in human gastric cancer and non-tumor gastric tissue

cytosolic extracts were quantified using a “Quantikine” human VEGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Cytosols were stored at -80℃ before measurement of VEGF levels. Diluted cytosols were incubated in triplicates overnight at 4℃ on microtiter plates coated with a murine monoclonal antibody against human VEGF. Unbound proteins were washed off, and an enzyme-linked polyclonal antibody specific for VEGF was added to “sandwich” the VEGF immobilized during the first incubation. A substrate solution for horseradish peroxidase was added, and color developed in proportion to the amount of antibody-bound VEGF. The absordance of the color was read at 450nm. A standard curve, consisting of known amounts of VEGF, was carried through the above procedure, and the concentrations of VEGF in the unknown samples were determined from this standard curve. Concentrations of VEGF were expressed as picograms per milligram cytosolic protein.

Concentrations of PlGF in human gastric cancer and non-tumor gastric tissue cytosolic extracts were quantified using a “Quantikine” human PlGF immunoassay (R&D Systems, Inc., Minneapolis, MN). The procedure was the same as that performed for VEGF.

Quantification of VEGF and PlGF was expressed as protein levels in human gastric cancer using EIA and ratios of VEGF and PlGF levels of gastric cancers to those of

non-tumor gastric mucosa.

Statistics

Expression difference of PlGF and VEGF between non-tumorous and tumorous tissues was determined by Wilcoxon sum rank test. The relationship between PlGF, VEGF, and the various clinicopathological factors was examined by Wilcoxon sum rank test. A multivariate analysis for correlation between PlGF, VEGF, clinicopathological factors and MVD was performed by linear regression method. Correlation between PlGF, VEGF expression and tumor stages was determined by Kruskal Willis test. Univariate survival analysis was calculated with Kaplan-Meier method, and the differences were analyzed by the Log-rank test. A multivariate survival analysis was performed using Cox proportional hazards model to investigate the independent prognostic factors. Statistical significance was defined as $p < 0.05$.

Results

VEGF and PlGF protein levels in gastric adenocarcinoma

Protein levels of VEGF and PlGF in gastric cancer tissue ranged from 1.2 pg/mg to 672.7 pg/mg, and 1.6 pg/mg to 239 pg/mg, and the median value was 66.7 pg/mg and 48.5 pg/mg, respectively. Protein levels of VEGF and PlGF in corresponding non cancerous mucosa ranged from 4.2 pg/mg to 549.5 pg/mg, and 0.7 pg/mg to 58.5 pg/mg, and the median value was 80.7 pg/mg and 9.8 pg/mg, respectively. PlGF protein levels were significantly higher than in most neoplasm in the corresponding non-tumorous mucosa ($p=0.000$). In contrast, VEGF protein levels were similar to those in the corresponding non-tumorous mucosa ($p=0.522$)(Fig. 1).

Localization of VEGF and PlGF in the gastric adenocarcinoma tissues

Immunohistochemical studies of gastric cancer tissues revealed that both PlGF and VEGF were localized in the cytoplasm of cancer cells. In addition to cancer cells, VEGF immunoreactivity was also present in some fibroblasts, smooth muscle cells, inflammatory cells and vascular endothelial cells (Fig. 2).

Correlation between PlGF or VEGF overexpression ratios and microvessel density and clinicopathologic factors

One of aims of this study was to examine correlation of MVD and expressions of VEGF and PlGF in human gastric cancer. It was found that there was a significant

correlation of PlGF expression with MVD but not of VEGF with MVD (Pearson correlation coefficient r=0.246, p=0.037 and r=0.076, p=0.526, respectively). Expression level of PlGF was not correlated significantly with that of VEGF (Pearson coefficient r=-0.048, p=0.686).

Over-expression levels of VEGF and PlGF standardized by a ratio of tumor to non-tumor tissue ranged from 0.01 to 82.43 and 0.10 to 58.62, respectively. The ratio of VEGF and PlGF that was greater than one was observed in 48.1% (38/79) and 88.6% (70/79) of patients, respectively. The median values for VEGF and PlGF of these patients were 1.160 and 5.640, respectively. Therefore, we classified them into two subgroups: high VEGF and PlGF group, for which the ratios were 1 and 6, respectively, or greater, and low VEGF and PlGF group, for which the ratios were 1 and 6, respectively. These two cutoff points were used to perform further statistical calculation correlated with clinicopathologic factors and survival.

Table 1 shows the correlation between VEGF and PlGF overexpression ratios, with MVD and various clinicopathological factors. VEGF of intestinal type gastric cancer was significantly higher than that of diffuse type gastric cancer (p=0.033). There was no other significantly association between VEGF, MVD and clinicopathological factors as tested in this study. However, PlGF in the patients with T3 and T4 lesions, positive lymph node metastases, and greater MVD (>32) was significantly higher than

in those with T1 and T2 lesions (p=0.026), negative lymph node metastases (p=0.002), less MVD (<32). A multiple linear regression analysis showed only PlGF was significantly correlated with MVD (p=0.0228) (Table 2). PlGF was significantly associated with tumor stages (p=0.011), but VEGF wasn't (p=0.586)(Fig 3).

Correlation between PlGF or VEGF overexpression ratios and survival

The survival rates were calculated using the Kaplan-Meier method. The survival rate of the group with high PlGF was significantly lower than that with low PlGF (30.5% v.s. 51.4%, p=0.0475), however, VEGF didn't show significant difference of survival rate (37.5% v.s. 45.7%, p=0.6053) (Table 3). The effects of variables presumably associated with patient survival were studied by multivariate analysis using Cox proportional hazards model. As a result, MVD and depth of tumor invasion were independent prognostic factors in this study (Table 4).

Correlation between PlGF or VEGF protein levels in gastric cancer with clinical outcome

For comparison with overexpression ratio, protein levels of PlGF and VEGF of gastric cancer were used to perform statistical calculation. Results of correlation study using PlGF and VEGF protein levels in gastric cancer were consistent with those using PlGF and VEGF overexpression ratios. Table 5 shows the correlation between VEGF and PlGF expression ratios, with MVD and various clinicopathological factors.

VEGF of intestinal type gastric cancer was significantly higher than that of diffuse type gastric cancer (p=0.017). There was no other significantly association between VEGF, MVD and clinicopathological factors as tested in this study. However, PlGF in the patients with T3 and T4 lesions, positive lymph node metastases, and greater MVD (>32) was significantly higher than in those with T1 and T2 lesions (p=0.026), negative lymph node metastases (p=0.022), less MVD (<32) (p=0.029). PlGF expression level was significantly associated with tumor stages (p=0.020), but VEGF wasn't (p=0.219)(Fig 4). Using a protein level that defined the upper thirty percent with the highest PlGF protein level (>120 ng/mg) showed significant survival difference (p=0.0470). VEGF protein level can't show any survival difference.

Conclusion

In conclusion, upregulated PlGF expression was found in ninety percent of gastric cancers and was strongly associated with MVD-evaluated angiogenesis, which was an independent prognostic factor in this study. PlGF may represent a possible antiangiogenic target for treatment of patients with gastric cancer.