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Neoangiogenesis in Patients with Gastric Carcinoma in Relation to the Expression of Vascular Endothelial Growth Factor and Thymidine Phosphorylase

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Abstract. *Background: We investigated the prognostic significance of microvessel density and the relationship between the expression of vascular endothelial growth factor (VEGF) and thymidine phosphorylase (TP), and angiogenesis in patients with gastric carcinoma. Materials and Methods: The expression of VEGF and TP, and the microvessel density were examined by immunohistochemistry in patients with gastric carcinoma invading the serosa. Results: The prognosis of patients with low microvessel density in the cancerous tissue was significantly better than that of patients with high microvessel density. A multivariate analysis showed that microvessel density, lymph node metastasis and tumor size were independent prognostic indicators. VEGF was expressed in tumor cells and TP was expressed in both tumor cells and infiltrating cells. VEGF expression in tumor cells and TP expression in infiltrating cells significantly correlated with microvessel density. However, microvessel density was not correlated with TP expression in tumor cells. Combined analysis based on VEGF expression in tumor cells and TP expression in infiltrating cells revealed that microvessel density was the highest in VEGF-positive and TP-positive tumors and the lowest in VEGF-negative and TP-negative tumors. Microvessel density is an independent prognostic indicator in patients with gastric carcinoma invading the serosa. Conclusion: VEGF expression in tumor cells and TP expression in infiltrating cells may indicate the microvessel density.*

It is known that malignant tumors depend on neovascularization for their growth and metastasis (1, 2). It has also been suggested that the degree of tumor angiogenesis is related to clinical outcome in patients with breast carcinoma (3), non-small cell lung carcinoma (4), gastric carcinoma (5), esophageal carcinoma (6), colon carcinoma (7) and cervical carcinoma (8). Angiogenesis is thought to be regulated by positive regulators and negative regulators that are secreted by both host and tumor cells (1, 9). Among these regulators, vascular endothelial growth factor (VEGF) and thymidine phosphorylase (TP) are thought to be major positive angiogenesis regulators (10,11). Maeda *et al.* reported a close correlation between VEGF expression, TP expression in carcinoma cells and tumor vascularity in cases of gastric carcinoma (12). However, TP expression in infiltrating cells was not evaluated in that study. Takahashi *et al.* reported that TP expression in infiltrating cells closely correlated with tumor vascularity in cases of colon carcinoma (13). It is still unclear which expression of angiogenic factors in various cells is most important for neoangiogenesis in gastrointestinal tract carcinomas.

Thus, in the current study, the relationship between the microvessel density and the expression of VEGF and TP in carcinoma cells and infiltrating cells was examined by immunohistochemistry in patients with gastric carcinoma invading the serosa who had undergone curative resection.

Materials and Methods

Patients. A total of 114 patients with advanced gastric adenocarcinoma with serosal invasion, who had undergone curative gastrectomy at our institution from 1986 to 1995, were used in this study. There were 61 male and 53 female patients. Their ages ranged from 24 to 91 years (mean, 60.3 years). The clinicopathological findings were determined according to the rules set by the Japanese Gastric Cancer Association (14). The criteria considered for curative resection were as follows: the complete removal of a primary gastric tumor, dissection of regional lymph nodes and no remaining macroscopic tumor. These patients had no metastasis in the liver, peritoneum or in other distant organs at the time of surgery. No other previous or concomitant primary cancer was present and the patients had received neither chemotherapy nor radiation therapy before surgery. All patients included in the study had undergone either partial gastrectomy (more than two thirds of the stomach) or total gastrectomy with regional lymph node dissection to group 1 (D1), group 2 (D2) or group 3 (D3) with curative intent. None of the resection margins were positive for tumors. The patterns of

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recurrence were determined by reviewing the medical records, which included laboratory data as well as information about the following tests and procedures: X-ray films, double contrasted barium meal study, gastrointestinal endoscopy, ultrasonography, computed tomography, scintigrams, peritoneal punctures and laparotomy. When there was more than one pattern of recurrence, the most life-threatening site was considered as the main pattern.

Immunohistochemistry. Four-μm-thick sections were dewaxed in xylene, dehydrated in ethanol and then heated in a microwave oven (700W) for 10 minutes to retrieve the antigens. Endogenous peroxidase was blocked by incubation of samples in 3% hydrogen peroxide in methanol. After being washed with phosphate-buffered saline (PBS), the samples were incubated overnight with the first antibodies. Anti-VEGF polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was applied at a dilution of 1:200. This antibody is raised against a synthetic peptide corresponding to amino acidic residues 1 to 20 of human VEGF. It recognizes the 165, 189 and 121 amino splicing variants of VEGF. A detection procedure for TP expression was performed using anti-dThdPase monoclonal antibody obtained from Nippon Roche Research Center, Kanagawa, Japan, at a dilution of 1:500. This antibody was prepared with an antigen consisting of human dThdPase purified from a human colon cancer xenograft HCT116. The characterization of this antigen was reported by Nishida *et al.* (15).

Microvessels were detected by using anti-CD34 monoclonal antibody (Nichirei Ltd., Tokyo, Japan). The samples were then incubated with Envision-labeled polymer reagent (Dako Co., Ltd., Copenghagen, Denmark) for 60 minutes at room temperature. Envision-labeled polymer reagent is a peroxidase-labeled polymer conjugated to goat anti-rabbit and goat anti-mouse immunoglobulins in Tris-HCl buffer containing carrier protein and an anti-microbial agent. The reaction products were visualized with diaminobenzidine as the chromogen and the sections were counterstained with methyl green. Normal mouse immunoglobulin G was used instead of the primary antibodies for the negative controls.

For VEGF staining, smooth muscle in the sections served as a positive control, because smooth muscle cells have been shown to express VEGF. However, there was no VEGF staining in infiltrating cells. Immunoreactivity was graded as follows: positive, more than 10% of the carcinoma cells were stained; negative, no detectable expression or fewer than 10% of the carcinoma cells were stained (16). The expression of TP in carcinoma cells was graded as follows: slight, no detectable expression or fewer than 5% of the carcinoma cells were stained; moderate, more than 5% of carcinoma cells were stained; marked, more than 20% of carcinoma cells were stained. The degree of TP-positive infiltrating cells was graded as follows: slight, no or slight infiltration; moderate, infiltration into sections of the carcinoma tissue; marked, infiltration into almost all of the carcinoma tissue. TP expression in tumor cells or in infiltrating cells was considered as positive when moderate or marked infiltration of TP-positive cells was observed.

For detection of microvessels, a single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessels, tumor cells and other connective tissue elements. The counting procedure of microvessels is described in our previously published study (16). Briefly, the stained sections were screened at x 100 magnification (x 10 objective lens and x 10 ocular lens) under a light microscope (VANOX-S, OLYMPUS, Tokyo, Japan) to identify the five regions of the section with the highest number of microvessels. The image was visualized on a computer display (Macintosh 7500/100, Apple Computer Inc., Cupertino, CA, USA) through a color video camera module (XC-003, SONY, Tokyo, Japan) and color image freezer (AE-6905C, ATTO, Tokyo, Japan). Microvessels were counted in these areas at x 200 magnification (x 20 objective lens and x 10 ocular lens), and the average number of microvessels was recorded. The visualized area on the display was determined to be 0.075 mm^2. The mean value was used for the analysis. Large vessels with thick muscular walls were excluded from the count. The observation of the lumen was not required for the identification of a vessel. All of the histological slides were examined by two observers (ST and HS), who were unaware of the clinical data or the disease outcome. When the interpretation differed between the two observers, a conference microscope was used for re-evaluation and final decision.

Table I. *Microvessel density and clinicopathological features.*

Variables	Microvessel density		
	High (n=54)	Low (n=60)	*P* value
Age (years)	59.6 ± 12.9	61.0 ± 14.5	N.S. [a]
Gender			N.S.
Male (n=61)	29	32	
Female (n=53)	25	28	
Tumor location			N.S.
Upper (n=26)	9	17	
Middle (n=38)	21	17	
Lower (n=37)	17	20	
Whole (n=13)	7	6	
Size (cm)	8.1 ± 4.0	8.4 ± 3.9	N.S.
Histology			N.S.
Well (n=27)	14	13	
Poorly (n=87)	40	47	
Depth of invasion			N.S.
T3 (n=106)	48	58	
T4 (n=8)	6	2	
Lymph node metastasis			N.S.
Absent (n=69)	33	36	
Present (n=45)	21	24	
Lymphatic involvement			N.S.
Absent (n=41)	23	18	
Present (n=73)	31	42	
Vascular involvement	N.S.		
Absent (n=40)	15	25	
Present (n=74)	39	35	
Gross appearence			N.S.
1 (n=2)	0	2	
2 (n=31)	18	13	
3 (n=38)	16	22	
4 (n=23)	12	11	
5 (n=20)	8	12	
Gastric resection			N.S.
Partial (n=63)	30	33	
Total (n=51)	24	27	
Lymph node dissection			N.S.
D1 and D2 (n=50)	22	28	
D3 (n=64)	32	32	

[a] N.S., Not significant

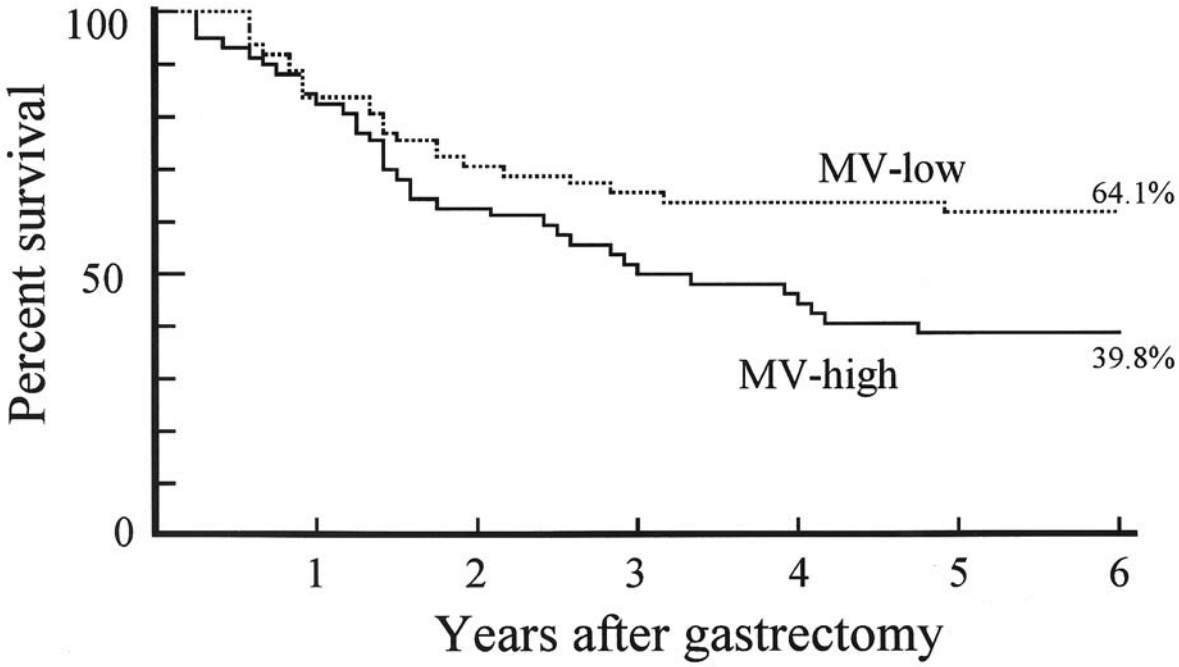

Figure 1. *Kaplan-Meier survival curves for patients with gastric carcinoma subdivided according to microvessel density (MV). The prognosis of patients with low microvessel density was significantly better than that of patients with high microvessel density.*

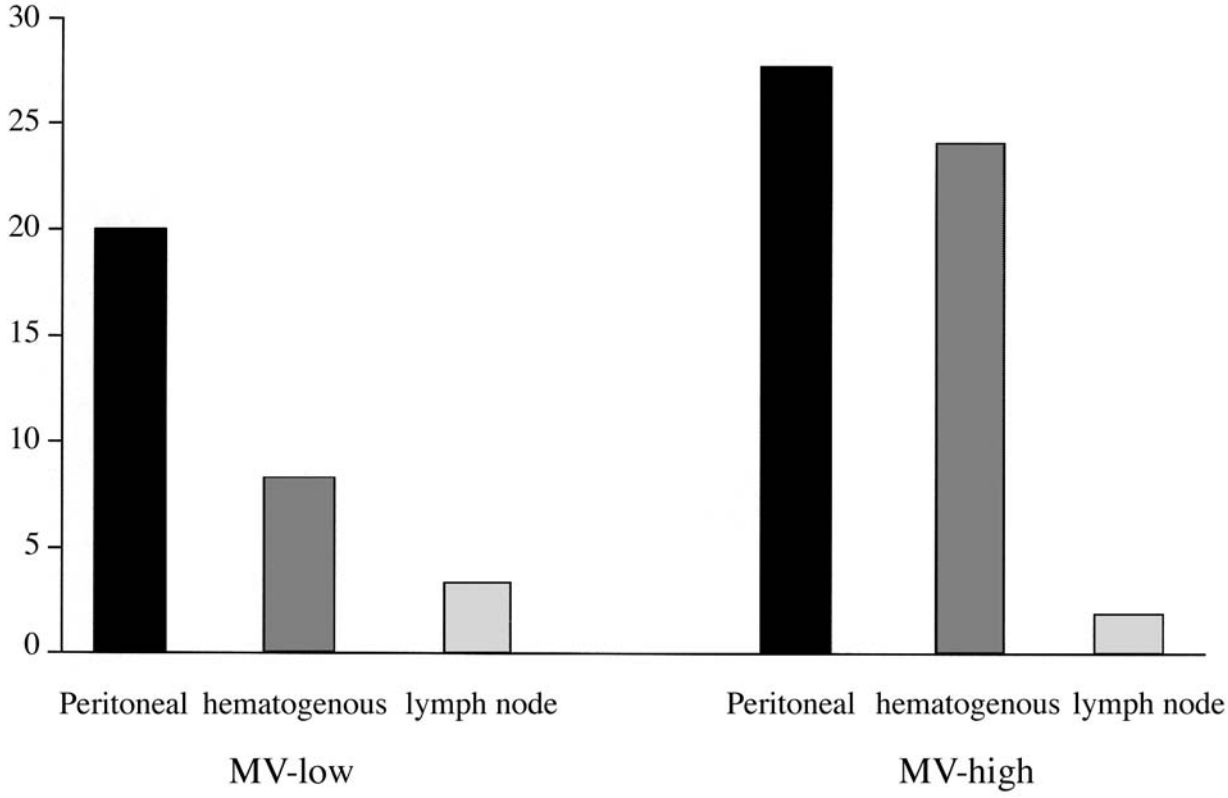

Figure 2. *The correlation between the mode of recurrence and microvessel density (MV). Microvessel density significantly correlated with hematogenous recurrence.*

Table II. *Association of various factors with overall survival determined by the Cox proportional hazards model and a stepwise procedure.*

Prognostic factors	*P*	Hazard ratio	95% CI
Lymph node metastasis (n_0-n_3) [a]	<0.0001	1.783	1.365 - 2.329
Tumor size [b]	0.0180	1.080	1.013 - 1.150
Microvessel density (high or low)	0.0092	2.102	1.201 - 3.677

[a] n_0, no regional lymph node metastasis; n_1, n_2 and n_3, metastasis in groups 1, 2 and 3 lymph nodes, respectively.
[b] continuous variables.

Table III. *Microvessel density in relation to the expression of VEGF and TP.*

	Microvessel density (mean ± S.D.)	*P* value
VEGF expression in tumor cells		$p<0.001$
negative (n=63)	37.3 ± 12.3	
positive (n=51)	53.0 ± 18.8	
TP expression in tumor cells		N.S.
slight (n=66)	41.9 ± 15.5	
moderate (n=20)	49.9 ± 26.4	
marked (n=28)	46.1 ± 12.2	
TP expression in infiltrating cells		$p<0.05$
slight (n=46)	42.0 ± 15.6	
moderate (n=34)	40.5 ± 12.2	
marked (n=34)	51.3 ± 21.8	

Statistical analysis. The association of factors was evaluated by the Chi-squared test. The significance of differences among means was determined by the Mann-Whitney (for two categories) and the Kruskal-Wallis (for three categories) tests. Differences between survival curves were examined with a log rank tests. The influence of each variable on survival was assessed by the Cox proportional hazards model and a stepwise procedure. The accepted level of significance was $p<0.05$. A Macintosh personal computer system (StatView software; Abacus Concepts, Inc., Berkeley, CA, USA) was used for all statistical analyses.

Results

Clinicopathological features and prognosis. The microvessel counts ranged from 13.8 to 136.4, with a mean value of 44.3 (standard deviation, 17.3). Patients were divided into two groups according to the microvessel counts, the cut-off value being 44.3 (the mean value for all of the patients). The correlation between microvessel density and clinicopathological features is presented in Table I. There was no correlation between the microvessel density and clinicopathological features. With regard to prognosis, the 5-year survival rate was 39.8% in patients with high microvessel density and 64.1% in patients with low microvessel density. Accordingly, the prognosis of patients with low microvessel density was significantly better than that of patients with high microvessel density (Figure 1).

Table IV. *Correlation between the expression of VEGF and TP.*

	VEGF-positive	VEGF-negative	*P* value
TP expression in tumor cells			N.S.
slight (n=66)	30	36	
moderate (n=20)	10	10	
marked (n=28)	11	17	
TP expression in infiltrating cells			N.S.
slight (n=46)	20	26	
moderate (n=34)	14	20	
marked (n=34)	17	17	

Table V. *Microvessel density in relation to the combination analysis of VEGF expression in tumor cells and TP expression in infiltrating cells.*

VEGF / TP status	Microvessel density (mean ± S.D.)
VEGF-positive / TP-positive (n=17)	59.7 ± 25.5
VEGF-positive / TP-negative or VEGF-negative / TP-positive (n=51)	47.2 ± 13.9
VEGF-negative / TP-negative (n=46)	35.3 ± 11.3

Mode of recurrence. A total of 53 patients experienced recurring disease. Peritoneal recurrence was observed most frequently (27 patients; 50.9%), followed by hematogenous recurrence (18 patients; 34.0%). Lymph node recurrence was observed in 3 patients (5.7%). In five patients, the recurrence sites were not determined in a single region. The rate of hematogenous recurrence in patients with high microvessel density was significantly higher than that of patients with low microvessel density (Figure 2).

Multivariate analysis. To assess whether the microvessel density represents a prognostic parameter, we used the Cox proportional hazards model and a stepwise procedure. The covariates included were age, gender, tumor size, histological classification, depth of invasion, lymph node metastasis, lymphatic vessel invasion, blood vessel invasion, type of gastrectomy, lymph node dissection and microvessel density. Age and tumor size were used as continuous

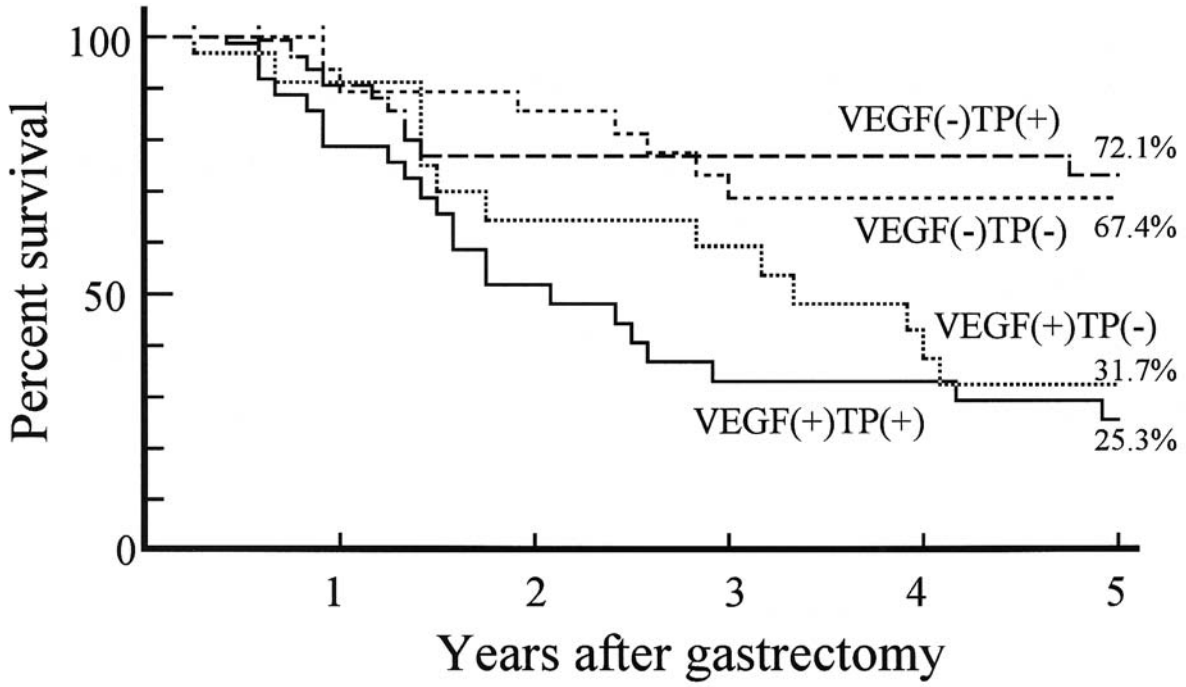

Figure 3. *Kaplan-Meier survival curves for patients with gastric carcinoma subdivided according to the expression of VEGF in tumor cells and TP in infiltrating cells. The prognosis of patients with VEGF-positive and TP-positive tumors was very poor as compared with the others.*

variables in this analysis. A multivariate analysis showed that lymph node metastasis was the strongest predictor of outcome. Moreover, microvessel density and tumor size were also independent prognostic indicators (Table II).

Expression of VEGF and TP. VEGF was localized mainly in the cytoplasm of the carcinoma cells. Tumor cells that were strongly immunopositive for VEGF were observed more often at the invasive front than in the center of the tumors. Weakly positive immunostaining for VEGF was seen on normal gastric mucosa and in some endothelial cells. VEGF expression was detected in 51 (44.7%) tumors. TP was distributed in the cytoplasm or in the nuclear compartments of the carcinoma cells and infiltrating cells *i.e.*, macrophages and histiocytes. Normal gastric mucosa was not immunoreactive with anti-dThdPase monoclonal antibody. TP expression in tumor cells was slight in 66 cases (57.9%), moderate in 20 cases (17.5%) and marked in 28 cases (24.6%). Moreover, TP-positive infiltrating cells was slight in 46 cases (40.4%), moderate in 34 patients (29.8%) and marked in 34 patients (29.8%).

The correlations between the microvessel density and VEGF or TP expression in carcinoma cells and infiltrating cells are given in Table III. Microvessel density was significantly correlated with VEGF expression in tumor cells and TP expression in infiltrating cells. Although gastric carcinomas with positive TP expression in tumor cells showed higher microvessel density than those with negative TP expression, the difference was not statistically significant.

The relationship between VEGF expression and TP expression is depicted in Table IV. There was no correlation between tumoral VEGF expression and TP expression in tumor cells or in infiltrating cells. The combined analysis of the expression of VEGF in tumor cells and TP in infiltrating cells with regard to microvessel density is shown in Table V. VEGF-positive and TP-positive tumors showed the highest microvessel density and VEGF-negative and TP-negative tumors showed the lowest microvessel density. Microvessel density of cases with the other expression patterns (VEGF-positive and TP-negative, or VEGF-negative and TP-positive) was between those two groups. The combined analysis of these two factors significantly correlated with microvessel density ($p<0.001$). Furthermore, the survival time of patients with VEGF-positive and stromal TP-positive tumors was very short as compared with the others (Figure 3).

Discussion

Gastric carcinoma is one of the most common malignancies in Japan. Many previous studies have shown that increased vascularity is associated with hematogenous recurrence (5) and poor prognosis (10, 11, 16, 17) in cases of gastric carcinoma. However, microvessel density was significantly correlated with the stage of disease (17). Most patients who had a recurrence were in the advanced stages of the disease. In the current study, patients with gastric carcinoma invading the serosa, who had undergone curative resection, were evaluated in terms of

the prognostic significance of microvessel density. The prognosis of patients with low microvessel density was significantly better than that of patients with high microvessel density. Moreover, a multivariate analysis indicated that microvessel density was an independent prognostic factor. Therefore, microvessel density appears to be a prognostic factor, irrespective of the stage of disease.

The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenetic factors that are produced by both tumor cells and normal cells. Numerous angiogenic factors have already been described. Of these angiogenic factors, VEGF and TP seem to play important roles in angiogenesis in cases of gastric carcinoma. VEGF is a *Mr* 34,000-50,000 dimeric glycoprotein synthesized by both tumor cells and normal cells (18). It consists of four isoforms that have 121, 165, 189, or 206 amino acid residues (19, 20). All four of these forms induce the mitogenesis of vascular endothelial cells and enhance vascular permeabilization. Previous reports have shown that VEGF acts as a paracrine mechanism through specific receptors on the surface of endothelial cells, flt-1 and KDR (21, 22). Previous studies have demonstrated that VEGF expression is a significant predictor of an increased risk of metastatic disease as well as a predictor of overall survival due to possible stimulation of angiogenesis in cases of gastric carcinoma (23), esophageal carcinoma (24), breast carcinoma (25), non-small cell lung carcinoma (26) and ovarian carcinoma (27). We have reported that VEGF expression is a sigificant predictor of an increased risk of hematogenous metastasis because it stimulates angiogenesis in cases of advanced gastric carcinoma (16). Although we observed a close correlation between VEGF expression and microvessel density in cases of human gastric carcinoma, several tumors observed in this study exhibited very high vessel density but had relatively low levels of VEGF. To determine whether another angiogenetic factor was responsible for this increase in angiogenetic activity, we determined the role of TP in human gastric carcinoma angiogenesis.

TP, an enzyme involved in pyrimidine nucleoside metabolism, has been demonstrated to be identical to platelet-derived endothelial cell growth factor (PD-ECGF) (28). PD-ECGF has been demonstrated to induce chemotaxis of endothelial cells *in vivo*, but it does not stimulate the growth of endothelial cells *in vitro*. TP expression is elevated in several solid tumor types. In a study of colon carcinoma, Takahashi *et al.* reported that TP expression in infiltrating cells closely correlated with tumor vascularity (17). Thus, the angiogenetic effects of TP expression in infiltrating cells and in tumor cells were determined in the current study. Although the microvessel density in tumors with cells positive for TP expression were higher than that in tumors with negative TP expression, the difference was not statistically significant. TP expression in tumor cells seemed to weakly correlate with angiogenesis. On the other hand, TP expression in infiltrating cells significantly correlated with angiogenesis. The observation that infiltrating cells may contribute to angiogenesis in solid malignancies was initially made by Polverini and Leibovich (29) and Leibovich *et al.* (30) in both *in vivo* and *in vitro* systems. Those studies demonstrated that tumor-associated macrophages and their conditioned media induce neovascularization in the cornea. In addition, those reports suggest that tumor-associated macrophages induce a stronger angiogenic response than do peritoneal macrophages; the authors discovered that conditioned media from tumor-associated macrophages induce a 10-fold increase in endothelial cell proliferation.

The effect of TP on angiogenesis may not be direct but may be *via* another angiogenic factor or through facilitation of endothelial cell invasion, due to its enzymatic activity. It is interesting to note that the highest vessel counts were observed in those tumors expressing both VEGF in tumor cells and TP in the infiltrating cells. Toi *et al.* observed similar findings in primary breast cancers (31). In that series, tumors with the highest vessel counts expressed both VEGF and TP in 75.5% of the cases. It is possible that the activity of one factor facilitates the angiogenic activity of the other factor. For example, the chemotactic activity of TP may facilitate tubule formation after the proliferation of endothelial cells occurs; this effect would be secondary to VEGF activity. Alternatively, the same conditions may induce the high expression of both of these angiogenic proteins, thereby inducing a strong angiogenic response. For example, it has been demonstrated that hypoxia induces the expression of both VEGF and TP (32). Similarly, it is possible that specific cytokine and/or growth factors may induce both VEGF and TP (33, 34). In the current study, however, there was no correlation between VEGF expression and TP expression either in tumor cells or in infiltrating cells. Whether TP and VEGF are redundant, additive, or synergistic remains to be determined.

In summary, high microvessel density is associated with poor prognosis in cases of gastric carcinoma with serosal invasion. Microvessel density is associated with VEGF expression in tumor cells and TP expression in infiltrating cells. Understanding the mechanisms of gastric carcinoma angiogenesis and the angiogenic phenotype provides a basis from which to approach the development of antiangiogenic therapies for patients with gastric carcinoma.

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