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High Level Expression of Platelet-derived Endothelial Cell Growth Factor predicts Good Prognosis in Colorectal Cancer Patients

Takashi TSUJI, Terumitsu SAWAI, Tohru NAKAGOE, Hiroshi YANO, Hirotoshi HASEBA, Hideaki KOMATSU, Hisakazu SINDOU, Hidetoshi FUKUOKA, Shigekazu HIDAKA, Shinichi SHIBASAKI, Atsushi NANASHIMA, Hiroyuki YAMAGUCHI, Tohru YASUTAKE and Hiroyoshi AYABE

First Department of Surgery, Nagasaki University School of Medicine

Platelet-derived endothelial cell growth factor (PD-ECGF) is one of the angiogenic factors. PD-ECGF expression is elevated in colorectal carcinoma, but its prognostic value does not reach a consensus. The aim of this study is to clarify the prognostic value of the PD-ECGF expression in colorectal carcinomas. PD-ECGF expression was measured by enzyme-linked immunosorbent assay in frozen materials from 71 colorectal cancer patients who had recived curative resection. Patients were divided into high expression and low expression groups based on cut-off value. Correlations among PD-ECGF expression, clinicopathologic features, and disease-free interval were studied by univariate and multivariate analysis. To evaluate the origin of PD-ECGF, serial sections of 71 tumors were stained for PD-ECGF and CD68. PD-ECGF expression in normal mucosa was 34.4 ± 15.5 (Units/mg protein) and the cut-off value was 65.4 (mean+2SD). There were no significant correlations between clinicopathological features and PD-ECGF expression. The disease-free interval for high PD-ECGF expression group was significantly longer than that of low expression group (P=0.006). A multivariate Cox's regression analysis revealed a high PD-ECGF expression as an independent factor for better outcome. In immunohistochemical study, almost all tumor cells were negative for PD-ECGF, but stromal macrophages were predominantly positive for PD-ECGF. In conclusion, the PD-ECGF expression measured in this study was derived from stromal macrophages. High PD-ECGF expression was a predictor for favorable outcome after curative resection for colorectal cancer.

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Address Correspondence: Takashi Tsuji, M.D.
First Department of Surgery, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki, 852-8501 Japan
TEL: +81-95-849-7304 FAX: +81-95-849-7306
E-mail: bannv@bolero.plala.or.jp

Introduction

Platelete-derived endothelial cell growth factor (PD-ECGF) is one of the angiogenic factors. PD-ECGF has chemotactic activity for endothelial cells in vitro and is angiogenic in vivo[1] PD-ECGF expression is elevated in several tumor types, including carcinomas of the breast[2], stomach[3], esophagus, lung, pancreas[4], and colorectum.

In colorectal carcinomas, although some reports suggest that high PD-ECGF expression in tumor cells is related to poor prognosis[5,6], but another shows that high expression in tumor stromal cells predicts a significantly better prognosis[7]. Thus, there is yet no consensus regarding whether PD-ECGF expression is a prongostic factor in colorectal carcinomas.

The aim of this study is to clarify whether PD-ECGF expression is an independent prognostic factor in patients with colorectal carcinoma. We also estimated the origin of PD-ECGF in colorectal carcinoma using immunohistochemical staining.

Materials and Methods

Subjects

We studied 71 patients with colorectal cancer who underwent curative resection at First Department of Surgery, Nagasaki University School of Medicine from February 1994 to December 1999. The mean age of patients was 64 years (range, 30-83) and 34 patients were male and 37 were female. No patients had been given preoperative chemotherapy or radiotherapy. Thirty-nine tumors were located in the colon and 32 tumors were localized in the rectum. Pathological classification, including stage, depth of invasion, histological type, lymphatic invasion, venous invasion and lymph node metastasis was defined in accordance with the General Rules for Clinical and Pathological Studies

on Cancer of the Colon, Rectum and Anus by the Japanese Society for Cancer of the Colon and Rectum[8]. Twelve tumors were classified as well-differentiated adenocarcinomas, 54 tumors as moderately differentiated adenocarcinomas, 2 tumors as poorly differentiated adenocarcinomas, 1 tumor as signet ring cell carcinoma, 1 tumor as mucinous carcinoma, and 1 tumor as undifferentiated carcinoma. The seventy-one patients included 13 patients in stage Ⅰ, 25 in stage Ⅱ, 18 in stage Ⅲa, 12 in stage Ⅲb, and 3 in stage Ⅳ. The median follow-up period was 42 months (minimum, 4.2 months). Sixty patients had received oral adjuvant chemotherapy during more than 1 year after surgery (HCFU 50, UFT 10). The written informed consents were obtained from all patients.

Enzyme-linked immunosorbent assay for Platelet-derived endothelial growth factor

Total protein extraction from frozen tissues was performed as follows. Frozen materials from primary tumor and normal mucosa stored at -80℃ were minced by scissors in a micro tube with extraction buffer containing a cocktail of three protease inhibitors (1 μg/ml of aprotinin and leupeptin, and 0.1mM PMSF) and were homogenized by kontes tube with pestle for 1 minute. The solution was centrifuged at 55000g for 45 minutes at 4℃. The supernatant was used for further analysis. Total protein concentration was analyzed with Bio-Rad protein assay kit (Bio-Rad, Tokyo, Japan). The PD-ECGF expression was measured by enzyme-linked immunosorbent assay (ELISA). The amount of PD-CEGF sandwiched with the two anti-PD-ECGF monoclonal antibodies (clone 104B and 232-2) was estimated by measuring its absorbency at 450 nm. The amount of PD-ECGF was calibrated with that measured for the standard solutions[9].

Immunohistochemical staining of PD-ECGF and CD 68

To evaluate the origin of PD-ECGF, 4 μm serial sections of formalin fixed paraffin embedded tissues of 71 tumors were stained for PD-ECGF and CD68. Anti-PD-ECGF monoclonal antibody[9] (clone 654-1) was donated by Nippon Roche Research Center, Kamakura, Japan. For visualizing a macrophage, anti-CD68 monoclonal antibody (clone KP1, DAKO, Japan) was used. Details for staining methods were described previously[7].

Statistical analysis

Statistical analyses were performed using the computer program STATISTICA (StatSoft, Tulsa, OK, USA). Fisher's exact probability test or Chi-square test was used for univariate analysis of categorical data and unpaired Student's-t test was applied for that of consecutive data. To estimate the prognostic value of PD-ECGF expression, the disease-free interval in 71 patients was studied. The Kaplan-Meier method[10] was used to estimate disease-free survival rate and difference between survival curves was tested for significance using log-rank test[11]. Multivariate analysis was performed with a Cox's proportional hazard regression model in order to assess the effects of different variables on patient[12]. A P value < 0.05 was considered to indicate statistical significance.

Results

Clinicopathologic features and PD-ECGF expression level

The mean value and standard deviation (SD) of PD-ECGF in normal colonic mucosa and in carcinoma was 34.4 ± 15.5 (range, 16.5-79.2) and 90.9 ± 52.4 (range, 16.3-250.2) (Units/mg protein), respectively. Tumors with synchronous liver metastasis or peritoneal metastasis tend to show low PD-ECGF expression, but there were no significant correlations between clinicopathological features and tumor PD-ECGF expression (Table-1).

Survival analysis and PD-ECGF expression

Because the pattern of PD-ECGF expression in normal mucosa showed normal distribution, we set up the cut-off value of tumor PD-ECGF at 65.4 (mean value of normal mucosa + 2SD). The high PD-ECGF expression was found in 47 cases (66%) and low expression was detected in 24 cases (34%). There were no significant differences in clinicopathologic features between the high and low PD-ECGF expression groups (Table-2). Thirty-nine of 47 patients (83%) with high PD-ECGF expression and 21 of 24 patients (86%) with low PD-ECGF expression had received adjuvant chemotherapy. Figure 1 shows the disease-free interval of patients with colorectal cancer according to the tumor PD-ECGF expression. Patients with high PD-ECGF expressions had longer disease-free intervals than those with low PD-ECGF expressions (P=0.006).

Hematogenous recurrence and PD-ECGF expression

Among 71 patients, 15 cases had experienced recurrence: hematogenous recurrence in 7, peritoneal recurrence in 4, and lymph node / local recurrence in 4 patients. The frequency of hematogenous recurrence was

Table 1. Relation between clinicopathologic features and PD-ECGF expression.

	No. of cases	PD-ECGF (Unit/mg protein)	*P* value
Depth of invasion			
sm	5	92.1 ± 47.3	n.s.*
mp	11	77.5 ± 37.7	
ss	33	91.2 ± 52.9	
se	17	110.0 ± 61.2	
si	5	52.1 ± 29.1	
Histological type			
well, mod	66	90.6 ± 54.1	0.88
por,muc,sig,undiff	5	94.4 ± 21.2	
Lymphatic invasion			
Absent	12	90.3 ± 38.4	0.96
Present	59	91.0 ± 55.0	
Venous invasion			
Absent	27	98.5 ± 46.2	0.34
Present	44	86.2 ± 55.8	
Lymph node metastasis			
Absent	41	87.2 ± 47.6	0.50
Present	30	95.8 ± 58.7	
Liver metastasis			
Absent	69	92.6 ± 52.1	0.10
Present	2	30.7 ± 5.0	
Peritoneal metastasis			
Absent	69	92.2 ± 52.4	0.21
Present	2	45.0 ± 25.2	
Stage			
I	13	84.0 ± 44.2	n.s.
II	25	97.3 ± 48.3	
IIIa	18	89.1 ± 67.6	
IIIb	12	99.8 ± 46.6	
IV	3	41.4 ± 18.9	

*n.s., not significant.

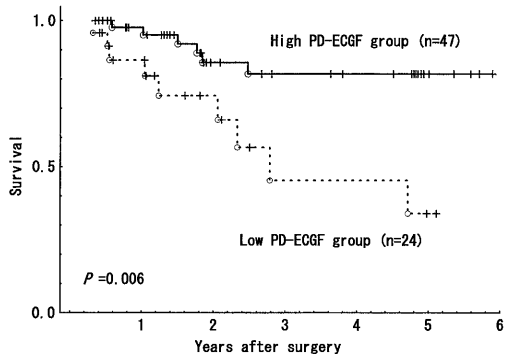

Figure 1. Disease-free interval after curative surgery for patients with colorectal cancer according to PD-ECGF expression.

Table 2. Comparison of clinicopatologic features between high and low PD-ECGF expression groups.

Variables	High group (n=47)	Low group (n=24)	*P* value
Sex			0.21
Male	25	9	
Female	22	15	
Age at operation			0.80
<65	24	13	
≦65	23	11	
Location			0.68
Rectum	22	10	
Colon	25	14	
Maximum size			0.45
<5 cm	24	10	
≦5 cm	23	14	
Macroscopic type			0.08
0, 1, 2	45	20	
3, 4, 5	2	4	
Histological type			0.10
well, mod	42	24	
por, muc, sig, undiff	5	0	
Depth of invasion			0.72
sm, mp	10	6	
ss, se, si	37	18	
Lymph node metastasis			0.11
Absent	24	17	
Present	23	7	
Lymphatic invasion			0.48
Absent	9	3	
Present	38	21	
Venous invasion			0.27
Absent	20	7	
Present	27	17	
Stage			0.07
I	7	6	
II	18	7	
IIIa	12	6	
IIIb	10	2	
IV	0	3	

Table 3. PD-ECGF expression and hematogenous recurrence.

PD-ECGF	Hematogenous recurrence (+)	Hematogenous recurrence (-)	*P* Value
High	2 (4%)	45 (96%)	0.04
Low	5 (24%)	19 (76%)	

significantly lower in high PD-ECGF group than in low PD-ECGF group (Table-3).

High PD-ECGF expression and standard prongostic factors for survival

In an univariate Cox's regression analysis, the survival of patients with colorectal cancers with high PD-ECGF expression was significantly better than that of patients with cancers with low PD-ECGF expression. Colorectal cancers with venous invasion were also associated with significantly lower survival (Table-4). In a step-down multivariate analysis, the PD-ECGF expression was found to be significantly and independently associated with disease-free survival (Table-5).

Table 4. Prognostic variables for disease-free survival in univariate analysis.

Variables and Categories	No. of cases	Hazard ratio (95% CI*)	*P* value
Sex			
Male/Female	34/37	0.96 (0.35-2.65)	0.93
Age at operation			
<65/65≦	37/34	0.84 (0.30-2.37)	0.75
Location			
Colon/Rectum	32/39	1.11 (0.39-3.13)	0.84
Maximum size			
<5cm/5cm≦	34/37	0.81 (0.29-2.24)	0.69
Macroscopic type			
0,1,2/3,4,5	65/6	2.99 (0.82-10.94)	0.10
Histological type			
well,mod/por,muc,sig,undiff	66/5	1.33 (0.30-5.90)	0.71
Depth of invasion			
sm,mp/ss,se,si	16/55	2.02 (0.71-5.79)	0.18
Lymph node metastasis			
Absent/Present	41/30	1.27 (0.46-3.52)	0.65
Lymphatic invasion			
Absent/Present	12/59	5.30 (0.69-40.53)	0.11
Venous invasion			
Absent/Present	27/44	3.79 (1.06-13.53)	0.04
Stage			
Ⅰ,Ⅱ,Ⅲa/Ⅲb,Ⅳ	56/15	2.64 (0.94-7.42)	0.07
PD-ECGF			
High/Low	47/24	4.13 (1.46-11.68)	0.007

*CI, Confidence interval.

Table 5. Prognostic variables for disease-free survival in multivariate Cox's regression analysis.

Variables	Categories	Hazard ratio	95% CI*	*P* value
PD-ECGF	High	1		
	Low	3.82	1.34-10.89	0.012
Stage	Ⅰ, Ⅱ, Ⅲa	1		
	Ⅲb, Ⅳ	2.36	0.82-6.78	0.110
Venous invasion	Absent	1		
	Present	2.80	0.77-10.26	0.120

*CI, Confidence interval.

Immunohistochemistry for PD-ECGF and macrophage

In immunohistochemical study, tumor cells were negative for PD-ECGF expression, with the exception of 3 cases (4.2%) in which a few tumor cells were

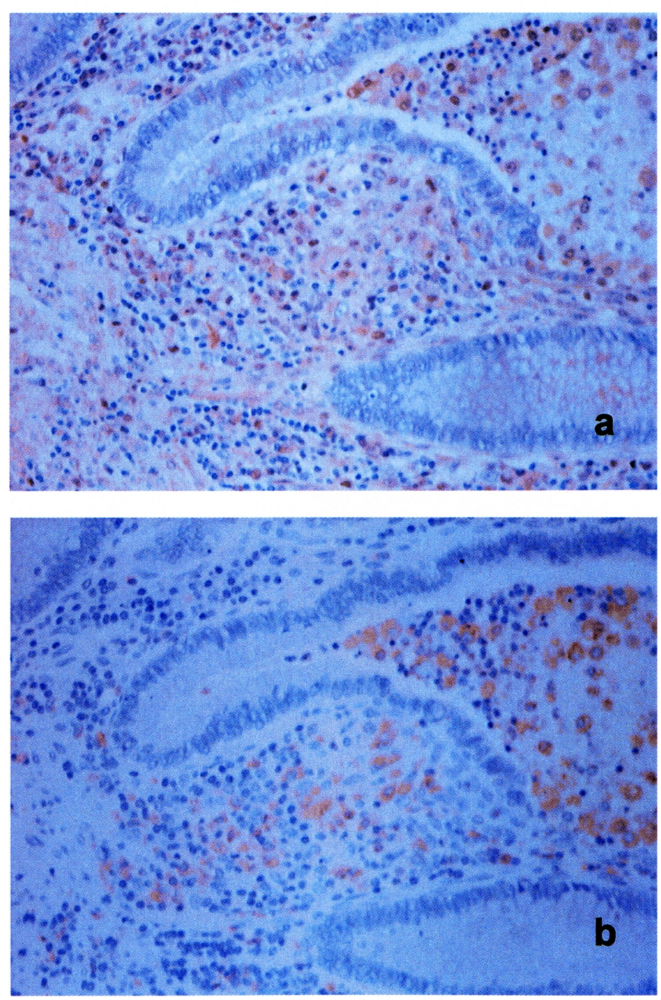

Figure 2. Immunohistochemical staining of colon carcinoma specimens with antibodies to (a) platelet-derived endothelial cell growth factor (PD-ECGF), and (b) CD68. (a) PD-ECGF expression predominantly was present in tumor stroma (counterstained with Mayer's hematoxylin, original magnification X200). (b) The staining pattern of CD68 was nearly coincident with that of PD-ECGF, suggesting that PD-ECGF positive cells predominantly were stromal macrophages (counterstained with Mayer's hematoxylin, original magnification X200).

stained. PD-ECGF positive cells predominantly were found in the stroma of tumor tissue (Fig. 2a). To characterize the PD-ECGF positive cells, sequential sections were immunostained with anti-human CD68 (KP1) antibody, a marker for human macrophages. The staining pattern of CD68 was nearly coincident with that of PD-ECGF (Fig. 2b).

Discussion

The prognostic value of the PD-ECGF expression in colorectal carcinoma does not reach a consensus. Some reports suggest that high expression of PD-ECGF in tumor cells is related to poor prognosis[5,6], but another shows that high expression in tumor stromal cells predicts a significantly better prognosis[7]. Because immunohistochemical study may be a little affected by subjectivity, we estimated PD-ECGF expression using ELISA.

In the current study, low PD-ECGF expression was related to hamatogenous metastasis and high PD-ECGF expression was found to be significantly and independently associated with better survival. PD-ECGF has been demonstrated to be identical to thymidine phosphorylase,[13] an enzyme involved in pyrimidine nucleoside metabolism that convert 5'-deoxy-5'fluorouridine and tegafur to 5-fluorouracil[14]. However, because the ratio of patients who had received adjuvant chemotherapy was not differ between high PD-ECGF expression group and low PD-ECGF expression group, adjuvant chemotherapy could not have an influence on the difference of their survival.

In immunohistochemical study of colorectal carcinoma, the main reason for the contradiction in prognostic value of PD-ECGF expression is a difference to evaluate the PD-ECGF expression in tumor cells or in stromal cells. Takebayashi et al.[5] estimated PD-ECGF stain in cancer cells and high expression was related to poor survival. On the other hand, in a recent report, Saito et al.[7] showed that high PD-ECGF expression in tumor stroma was associated with better survival. Our data is consistent with them. In present study, PD-ECGF positive cells were predominantly found in the stroma and most stromal macrophage were PD-ECGF positive in immunohistochemistry. One reason of this discrepancy in PD-ECGF staining pattern may be difference of monoclonal antibodies used. Takahashi et al.[15] reported that PD-ECGF expression originated from stromal macrophage using double-stainig method for PD-ECGF and CD68 in colorectal carcinomas. Recent study using immuno-electron microscopy for PD-ECGF indicated that the specific granules of the macrophages in the rectal cancer specimens showed positive for PD-ECGF, however, tumor cells showed diffuse stains[16].

What is function of macrophage infiltrating in tumors? It is suggested that tumor-infiltrating macrophages produce several angiogenic factors, such as vascular endothelial growth factor[17], fibroblast growth factor[18], and tumor necrosis factor-α[19]. Leek et al.[20] reported that significant correlation between high vascular grade and increased macrophage counts, and increased macrophage counts was associated with poor survival in breast cancer. On the other hand, tumor-infiltrating macrophages also produce the several angiogenic inhibitors, such as plasminogen activator-specific inhibitor[21] and thrombospondin 1[22]. Recently, Dong et al.[23] showed that tumor-infiltrating macrophages produce angiostatin which is inherent angiogenic inhibitor. A balance between angiogenic factors and angiogenic inhibitors in microenvironment may determine the effect of tumor-infiltrating macrophages on tumor angiogenesis.

The angiogenic effect of PD-ECGF is associated with 2-deoxy-D-ribose, catabolic product of thymidine by PD-ECGF/Thymidine phosphorylase[1]. The significance of PD-ECGF expression in tumor-infiltrating macrophages is yet unknown[24]. There may be differences in the functional activity of PD-ECGF produced by tumor cells and that produced by macrophages.

Macrophages are potent antigen-presenting cells, presenting antigens to helper T-cells, with the consequent development of an active cytotoxic T-lymphocyte reaction. A recent study showed that the presence of synchronous liver metastasis was correlated inversely with the number of macrophages distributed along the invasive margin of colorectal carcinoma[25].

In this study, we showed that stromal macrophages expressed PD-ECGF and high PD-ECGF expression was significantly and independently associated with longer disease-free interval in patients with colorectal carcinoma. Thus, it is recommended that colorectal cancer patients with low PD-ECGF expression must be followed up strictly.

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