Pergamon

European Journal of Cancer, Vol. 34, No. 9, pp. 1439–1447, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00069-0

Original Paper

Expression of Two Angiogenic Factors, Vascular Endothelial Growth Factor and Platelet-derived Endothelial Cell Growth Factor in Human Pancreatic Cancer, and its Relationship to Angiogenesis

K. Fujimoto, R. Hosotani, M. Wada, J.-U. Lee, T. Koshiba, Y. Miyamoto, S. Tsuji, S. Nakajima, R. Doi and M. Imamura

Department of Surgery and Surgical Basic Science, Kyoto University, 54 Shogoin-Kawara-cho, Sakyo-ku, Kyoto, 606-01 Japan

Tumour angiogenesis, as assayed by microvessel density (MVD), and the expression of vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) have become established as important prognostic indicators for many tumour types. In this study, MVD and the expression of VEGF and PD-ECGF were examined by immunohistochemical staining of 50 pancreatic cancer tissues, and the relationships between either MVD or the expression of these two angiogenic factors and the clinicopathological features, including survival, were analysed. The expression of VEGF and PD-ECGF proteins were confirmed by Western blot analysis and VEGF mRNA isoforms were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in five pancreatic cancer cell lines. Twenty-eight (56%) of 50 pancreatic cancers were positive for VEGF protein in cancer cells, and 16 (32%) showed strong PD-ECGF staining in cancer and infiltrating cells. $VEGF_{121}$ and $VEGF_{165}$ were identified as the predominant species produced in pancreatic cancer cells. The overexpression of VEGF and PD-ECGF protein significantly correlated with high MVD ($P = 0.002$, 0.044, respectively). Advanced stage of disease was significantly more frequent in patients with high MVD ($P = 0.025$). No significant association was found between the expression of VEGF or PD-ECGF and clinicopathological features, except for tumour histology. The expression of PD-ECGF correlated with poor survival ($P = 0.011$), but MVD and VEGF expression were not found to be useful for the prediction of overall survival. This study suggests that VEGF and PD-ECGF may play an important role in tumour angiogenesis, and that PD-ECGF expression seems to be useful for establishing prognoses for pancreatic cancer.

Key words: vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), angiogenesis, microvessel density, pancreatic cancer, RT–PCR, Western blot
Eur J Cancer, Vol. 34, No. 9, pp. 1439–1447, 1998

INTRODUCTION

NEOANGIOGENESIS IS a requirement for tumour growth and metastasis, and is induced by angiogenic growth factors produced by tumour cells and/or infiltrating cells [1]. Intratumoral microvessel density (MVD), a marker of angiogenesis grade, has been demonstrated to be a potent prognostic indicator in various types of malignant tumours [2–6]. In addition to immunohistochemical MVD analysis, the role of several angiogenic growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) in neovascularisation has also been investigated in human tumour specimens [2, 7–12]. Previously, we demonstrated that VEGF significantly contributes to angiogenesis of hepatocellular carcinoma, a hypervascular tumour, by evaluating the correlations between the degree of VEGF messenger RNA expression and arteriographic findings [11].

Pancreatic cancer is associated with very poor prognosis, with 5-year survival rates ranging from 0 to 18%, despite

Correspondence to K. Fujimoto.
Received 8 Oct. 1997; revised 22 Jan. 1998; accepted 29 Jan. 1998.

heroic attempts at resection [13]. Recently, Egawa and colleagues revealed that a potent angiogenesis inhibitor, O-(chloroacetylcarbamoyl) fumagillol (AGM-1470), specifically inhibited the growth of HPD-NR tumours *in vivo* through a decrease in the vascularity of the tumours, and showed that angiogenesis plays an important role in tumour growth in a Syrian hamster pancreatic cancer cell line HPD-NR, which closely resembles its human counterpart, and may represent a new target of medical therapy for pancreatic cancer [14]. However, as far as we are aware, no reports have been published describing the possible association between the expression of angiogenic factors and tumour angiogenesis in human pancreatic cancer.

Recently, Toi and associates demonstrated that VEGF and PD-ECGF are frequently coexpressed in human breast cancer specimens [15], whereas Takahashi and colleagues showed that VEGF and PD-ECGF act as mutually complementary factors in highly vascularised human colon cancer [10]. It remains unclear whether VEGF and PD-ECGF are in fact synergistic or complementary. Furthermore, several investigators have demonstrated that hypoxia enhances the expression of two angiogenic factors, VEGF and PD-ECGF [16, 17], and that ras oncoprotein is a potent inducer of VEGF mRNA, suggesting that persistent activation of signalling pathways induced by this oncoprotein account for overexpression of VEGF in human tumours [18–20]. Dynamic computed tomography (CT) and angiography have shown pancreatic cancers as low-density areas or regions with negative or slight enhancement, indicating hypovascularity of the tumour when compared with the surrounding normal pancreatic tissues [21]. Furthermore, numerous studies have shown that K-*ras* oncogene mutations are frequently identified in pancreatic cancer [22]. For these reasons, this study focused on these two angiogenic factors among a variety of angiogenic factors.

In order to determine whether VEGF and/or PD-ECGF is responsible for neoangiogenesis in human pancreatic cancer, we examined the correlation between their expression and intratumoral MVD in the present study. Furthermore, the relationships between either MVD within the tumour or the expression of these two angiogenic factors and clinicopathological features, metastasis or prognosis were also analysed.

MATERIALS AND METHODS

Patients and tumour samples

The study included 50 randomly chosen patients with pancreatic cancers who had undergone resection at the Department of Surgery and Surgical Basic Science of Kyoto University Hospital, Japan, between January 1987 and September 1996. The clinicopathological characteristics of 50 patients with ductal adenocarcinomas of the pancreas investigated in this study are summarised in Table 1. Patients with other pancreatic malignancies, such as intraductal papillary

Table 1. *Relationship between clinicopathological features and microvessel counts, vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) expression in pancreatic cancers* (n = 50)

		Microvessel counts†			VEGF expression			PD-ECGF expression‡		
Variables	No. of patients (%)	Hypervascular ($n = 21$)	Hypovascular ($n = 29$)	P*	Positive ($n = 28$)	Negative ($n = 22$)	P	Strong ($n = 17$)	Weak ($n = 33$)	P
Age (years)	62.4 ± 1.3	60.8 ± 2.0	63.5 ± 1.7	NS	60.9 ± 1.8	64.3 ± 2.0	NS	61.8 ± 2.3	62.7 ± 1.6	NS
Gender				NS			NS			NS
Male	28 (56)	13 (62)	15 (52)		14 (50)	14 (64)		7 (41)	21 (64)	
Female	22 (44)	8 (38)	14 (48)		14 (50)	8 (36)		10 (59)	12 (36)	
Tumour location				NS			NS			NS
Head	38 (76)	17 (81)	21 (72)		20 (71)	18 (82)		11 (65)	27 (82)	
Body or tail	12 (24)	4 (19)	8 (28)		8 (29)	4 (18)		6 (35)	6 (18)	
Histological type				NS			0.045			0.014
Well	9 (18)	3 (14)	6 (21)		2 (7)	7 (32)		0 (0)	9 (27)	
Moderately	31 (62)	15 (71)	16 (55)		21 (75)	10 (45)		13 (76)	18 (55)	
Poorly	10 (20)	3 (14)	7 (24)		5 (18)	5 (23)		4 (24)	6 (18)	
Lymph node metastasis				NS			NS			NS
Absent	21 (42)	6 (28)	15 (52)		10 (36)	11 (50)		6 (35)	15 (45)	
Present	29 (58)	15 (71)	14 (48)		18 (64)	11 (50)		11 (65)	18 (55)	
Hepatic metastasis§				NS			NS			NS
Absent	45 (90)	18 (86)	27 (93)		25 (29)	20 (91)		15 (88)	30 (91)	
Present	5 (10)	3 (14)	2 (7)		3 (11)	2 (9)		2 (12)	3 (9)	
pT (UICC)				NS			NS			NS
T1	2 (4)	1 (5)	1 (3)		1 (4)	1 (5)		0 (0)	2 (6)	
T2	14 (28)	5 (24)	9 (31)		8 (29)	6 (27)		4 (24)	10 (30)	
T3	34 (68)	15 (71)	19 (66)		19 (68)	15 (68)		13 (76)	21 (64)	
Stage (UICC)				0.025			NS			NS
I	6 (12)	0 (0)	6 (21)		2 (7)	4 (18)		1 (6)	5 (15)	
II	13 (26)	5 (24)	8 (28)		7 (25)	6 (27)		5 (29)	8 (24)	
III	15 (30)	6 (28)	9 (31)		7 (25)	8 (36)		5 (29)	10 (30)	
IV	16 (32)	10 (48)	6 (21)		12 (43)	4 (18)		6 (35)	10 (30)	

NS, no significant difference. *P values were obtained from the chi-squared test. †Hypervascular, patients with microvessel count ≥ mean value (= 30.1); Hypovascular, patients with microvessel count < mean value. ‡Strong, the sum of staining intensity of cancer and infiltrating cells was 4 or more; Weak, the sum of staining intensity of cancer and infiltrating cells was 3 or less. §Patients with hepatic metastasis at the time of operation.

mucinous adenocarcinomas, acinar cell carcinomas and endocrine tumours, were excluded. The average age at surgery was 62.4 years (range 41–89 years). At the time of surgery, pancreatic cancer was staged according to the pTNM (UICC) system [23]. All patients had survived at least 60 days after operation, to exclude perioperative mortality-related bias. Follow-up for the patients included in the survival analysis was updated on 1 September 1997 (median follow-up was 12.2 months [range 2.4–82 months]). At that time, 46 patients had died of pancreatic cancer and 4 were alive.

Histopathological diagnosis was confirmed at the Department of Pathology of Kyoto University Hospital. Tumour specimens were collected after obtaining informed consent in accordance with institutional guidelines, and immunohistochemical studies of VEGF, PD-ECGF and CD34 were performed.

Immunohistochemical staining

Samples were fixed with 4% paraformaldehyde or 10% formaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and cut into consecutive 4 μm thick sections. Immunohistochemical staining was performed by the immunoperoxidase technique. Paraffin sections were dewaxed in three changes of xylene, followed by rinsing in graded ethanol and finally rehydrated in three changes of PBS. Antigen retrieval for VEGF and PD-ECGF staining was performed by microwave treatment to unmask tissue antigens. For CD34, trypsin digestion was carried out to unmask the antigen, as this gave a more producible reaction than microwave treatment. Endogenous peroxidase was blocked by incubating the sections in 1% hydrogen peroxide in methanol for 15 min at room temperature. After rinsing three times with PBS for 5 min, non-specific reaction was blocked by incubating the sections with PBS containing 5% normal goat serum for 30 min at room temperature. The sections were then incubated with appropriate dilutions of the primary antibody at 4°C overnight. The antibodies used were a rabbit polyclonal immunoglobulin (IgG) antibody (A-20, Santa Cruz Biotechnology, California, U.S.A.) for VEGF at a 1:200 dilution, a mouse monoclonal IgG antibody (#2A4, from Y. Yamada, Taiho Pharmaceutical Co. Ltd, Hannou, Japan) [9, 24] for PD-ECGF at a 1:500 dilution and a mouse monoclonal IgG antibody (QB-END/10, Novocastra, Newcastle, U.K.) for CD34 at a 1:25 dilution. Anti-CD34 antibody was used for staining vascular endothelial cells and quantitating tumour vascularisation. An oesophageal cancer specimen known to express VEGF was used as a positive control of VEGF and a normal liver section was used as a positive control of PD-ECGF, as Kupffer cells express a high level of PD-ECGF [24]. Negative controls were performed using non-specific mouse or rabbit IgG as the primary antibody. After rinsing three times with PBS for 5 min, the sections were incubated with the appropriate peroxidase-labelled secondary antibody for 1 h at room temperature. After rinsing three times with PBS, the sections were incubated with diaminobenzidine substrate for 5 min. The sections were rinsed with distilled water and counterstained with Mayer's haematoxylin solution.

Evaluation of immunostaining and vessel counting

To evaluate the intensity of VEGF and PD-ECGF staining, groups of cells with the strongest staining within each tumour were examined, using the method described by Takahashi and colleagues [2]. The intensity of VEGF staining of cancer cells was graded on a scale of 0–3+: 0 = no staining of cancer cells; 1+ = weak staining; 2+ = moderate staining; and 3+ = strong staining. The intensity of staining for PD-ECGF was assessed in both cancer cells and infiltrating cells on a scale of 0–3+ as well as VEGF staining. The specimen was regarded as positive when the intensity of staining was 2+ or 3+, and negative when the intensity of staining was 0 or 1+.

Vessel counts within the tumour were assessed by light microscopy after staining for CD34. Based on the criteria of Weidner and associates [25], a vessel lumen was not required for identification of a microvessel. The three areas with the highest number of discrete microvessels were identified by scanning tumour sections at low power (×100). After the areas of highest neovascularisation were identified, photographs of each area were taken at ×200 magnification (×20 objective and ×10 ocular, corresponding to a 0.75 mm^2 area) to obtain accurate microvessel counts, and the average counts of the three areas were recorded.

The evaluation of VEGF and PD-ECGF expression, and microvessel counts were simultaneously performed by two investigators (K.F. and S.N.) without knowledge of the patients' clinicopathological features.

Cell lines and culture

Five human pancreatic cancer cell lines, namely PANC-1, AsPC-1, MIAPaCa-2, CFPAC-1 and Capan-2, purchased from the American Type Culture Collection (Rockville, Maryland, U.S.A.), were used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of VEGF mRNA isoforms and Western blot analysis of VEGF and PD-ECGF proteins. Culture conditions were as follows: cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) for PANC-1, in RPMI 1640 with 20% FCS for AsPC-1, Minimum Essential medium with 10% FCS for MIAPaCa-2, in Iscove's modified Dulbecco's medium supplemented with 10% FCS for CFPAC-1, and in McCoy's 5A with 10% FCS for Capan-2. All cells were cultured and treated at 37°C in a humidified incubator containing 5% CO_2.

RNA extraction and RT-PCR analysis

In order to perform RT-PCR analysis, total cellular RNA was prepared using TRIZOL Reagent (Life Technologies, Gaithersburg, Maryland, U.S.A.) and cDNA was prepared by random priming from 1 μg of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia Biotech, Sweden) according to the manufacturers' instructions. PCR was carried out with a mixture consisting of cDNA derived from 100 ng of RNA, 20 pmol each of upstream and downstream primers for the sequence of the *VEGF* gene, 200 μmol of deoxynucleotide triphosphate and 2.5 U of Taq DNA polymerase with reaction buffer (TaKaRa Ex Taq) in a final volume of 50 μl. PCR was performed for 30 cycles in a Thermal cycler (PTC-100, MJ Research, Inc., Massachusetts, U.S.A.) as follows: 1 min at 94°C for denaturation, 2 min at 60°C for annealing and 2 min at 72°C for extension. Oligonucleotide primers to amplify multiple *VEGF* transcripts were designed on the human gene sequence [8], upstream primer: 5′-GGATGTCTATCAGCGCAGCTAC-3′, downstream primer: 5′-TCACCGCCTCGGCTTGTCACATC-3′ to detect potentially all four *VEGF* mRNAs, giving origin to a

322 bp band in the case of $VEGF_{121}$, a 454 bp band in the case of $VEGF_{165}$, a 526 bp band in the case of $VEGF_{189}$ and a 577 bp band in the case of $VEGF_{206}$.

Protein extraction and Western blot analysis

Protein was extracted from cultured cell lines. Cultured cell lines were incubated for 20 min and sonicated twice for 5 sec in cold lysis buffer (10 mM PBS pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 40 µg/ml aprotinin. Total extracts were cleared by centrifugation for 30 min at 4°C at 14 000 rpm. Protein levels were quantified using the method of Bradford with bovine serum albumin (BSA) as the standard [26]. Samples were resolved by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). In all gels, 50 µg of protein dissolved in sample buffer was loaded per lane. Immunoblotting was performed by electroblotting on to PVDF membrane (0.2 µm) (Bio-Rad, California, U.S.A.) at 32 mA for 1 h using a semidry immunoblotter (Trans-Blot, Bio-Rad). Membranes were blocked for 2 h at room temperature in blocking buffer (150 mM NaCl, 20 mM Tris–HCl pH 8 (TBS) and 5% "Yukijirushi" fat-free milk powder) and subsequently incubated for 1 h at room temperature with anti-VEGF polyclonal antibody at a 1:200 dilution or anti-PD-ECGF monoclonal antibody at a 1:500 dilution with blocking buffer. The enhanced chemiluminescence system (Amersham Life Sciences, Amersham, U.K.) was used for the detection of bound antibody. Primary antibody-bound membranes were incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-rabbit or antimouse IgG at a 1:1000 dilution with blocking buffer. After washing with TBS and TBST (150 mM NaCl, 20 mM Tris–HCl pH 8 and 0.05% Tween 20), the membranes were treated with enhanced chemiluminescence reagents according to the manufacturer's protocol. The membranes were exposed to X-ray film for 5–60 sec.

Statistical analysis

Clinicopathological characteristics were compared between patients with high and low MVD, VEGF-positive and -negative tumours, and PD-ECGF-strong and -weak tumours with the use of the chi-squared test (or Fisher's exact probability test) or Student's *t* test. Comparisons between the microvessel count and VEGF or PD-ECGF staining were evaluated by Student's *t* test. The Kaplan–Meier method was used to calculate survival curves, and the log-rank test and the generalised Wilcoxon test were performed to compare differences in survival rates of the patient groups. All statistical analyses were performed using JMP statistical software (version 3.02) for the Macintosh. A value of $P < 0.05$ was deemed significant.

RESULTS

Microvessel counts (CD34) and its correlation with clinicopathological features

The microvessel staining for CD34 within the tumour was mainly found in the interstitum around the cancer cells with

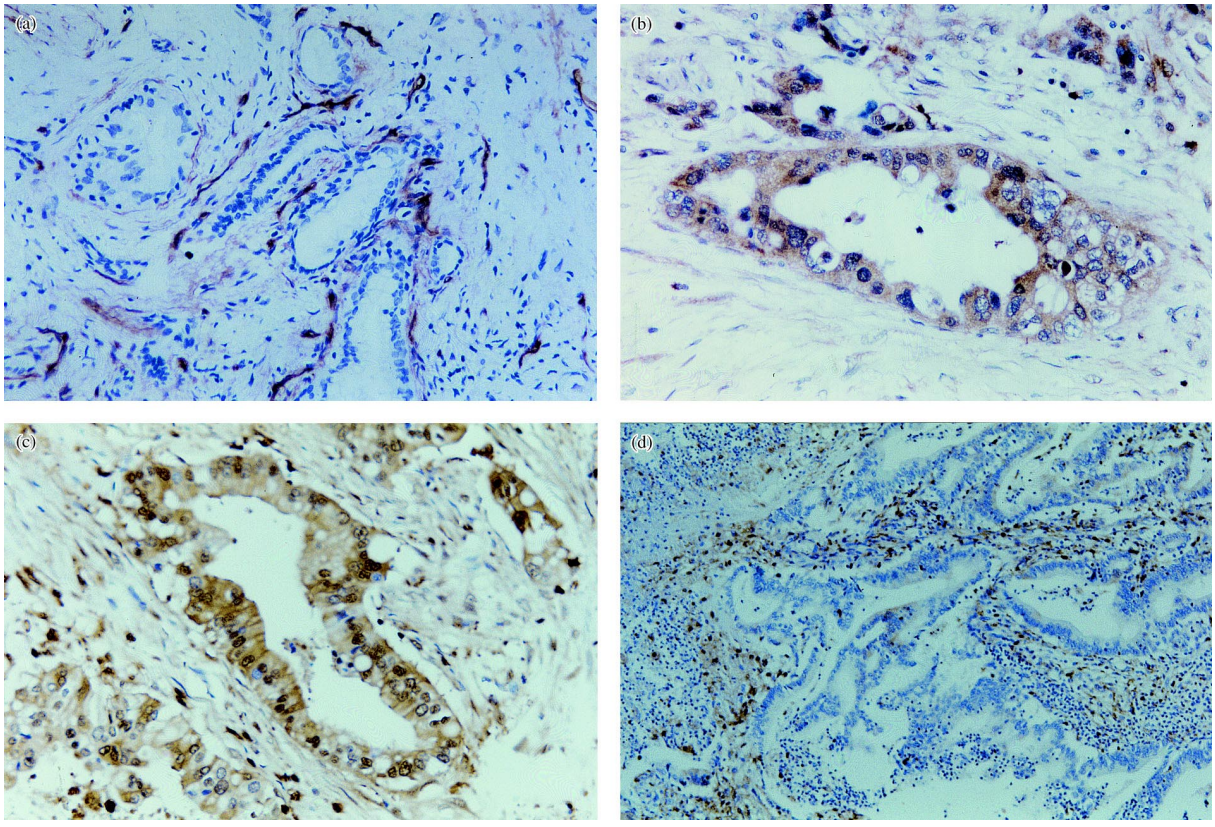

Figure 1. Immunohistochemical staining of microvessel (CD34) within the tumor (a), positive vascular endothelial growth factor (VEGF) expression (b), and strong platelet-derived endothelial cell growth factor (PD-ECGF) expression (c and d) in pancreatic cancer specimens. (c) Shows that positive staining for PD-ECGF is mainly observed in cancer cells. (d) Shows that positive staining for PD-ECGF is solely observed in infiltrating cells, neither type of cancer cells stained positive. (a)–(c) were photographed at 200× and (d) was photographed at 100×.

tubular formation (Figure 1a). The total microvessel counts ranged from 1 to 81 at a 200× microscopic high-power field (hpf) and the mean microvessel counts in 50 pancreatic cancer specimens was 30.1/hpf. When a mean value of 30.1 was chosen as the cut-off point for discrimination of the 50 patients into two subgroups, 21 patients were categorised as hypervascular and 29 as hypovascular. Among the clinicopathological variables examined, age, gender, tumour location, histological type, lymph node metastasis, hepatic metastasis and pT were equally distributed among these two subgroups (Table 1). However, advanced stage of disease was significantly more frequent in the hypervascular group ($P = 0.025$) (Table 1). When survival curves of the hypervascular and hypovascular groups were compared, the survival rates for 21 patients with hypervascular tumours and 29 with hypovascular tumours were not significantly different (Figure 2a).

Immunohistochemical analysis of VEGF and PD-ECGF expression in pancreatic cancer and normal sections

The staining of VEGF was mainly identified in the cytoplasm of cancer cells (Figure 1b). In normal tissue specimens, islet cells were clearly stained and some acinar cells in the peripheral regions of the lobes were weakly stained, although most acinar cells and ductal cells were not stained with the anti-VEGF antibody. Twenty-eight of 50 pancreatic cancers (56%) were positive for VEGF expression; the staining intensity scale was greater than 2.

Acinar cells and islet cells were not stained with PD-ECGF antibody in normal tissue specimens, although ductal cells were weakly stained. In the tumour specimens, the staining of PD-ECGF was identified in the cytoplasm and/or nucleus of cancer cells and in the infiltrating cells (Figure 1c and Figure 1d). Twenty-two of 50 pancreatic cancers (44%) were positive for PD-ECGF expression in cancer cells and 25 of 50 (50%) were positive in infiltrating cells. We performed a statistical analysis to determine whether there is any relationship between PD-ECGF reactivity in cancer cells and in infiltrating cells. Sixteen of 50 pancreatic cancers (32%) were positive for PD-ECGF expression in both cancer cells and infiltrating cells and 19 of 50 (38%) were negative in both. There was a significant correlation between PD-ECGF reactivity in cancer cells and in infiltrating cells ($P = 0.0044$). Because we considered that the positive staining of infiltrating cells was also meaningful, we divided the expression of PD-ECGF into two groups by summing the scales for cancer and infiltrating cells as follows: strong expression group = a sum of staining intensity of cancer and infiltrating cells was 4 or more; weak expression group = a sum of staining intensity of cancer and infiltrating cells was 3 or less. In this classification, 17 of 50 pancreatic cancers (34%) had strong PD-ECGF staining.

Table 2. Correlation between vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) expression

	VEGF expression		
	Positive	Negative	*P* value
PD-ECGF expression (cancer + infiltrating cells)			
Strong	14	3	0.0053
Weak	14	19	
PD-ECGF expression (cancer cells)			
Positive	17	5	0.0072
Negative	11	17	

One of the aims of this study was to examine an association of VEGF expression with PD-ECGF. As shown in Table 2, there was a highly significant correlation between VEGF expression and PD-ECGF expression in both cancer cells and infiltrating cells ($P = 0.0053$). Furthermore, we performed another statistical analysis to determine whether it is cancer cell PD-ECGF expression that is correlated with VEGF expression. There was also a significant correlation between VEGF expression and cancer cell PD-ECGF expression ($P = 0.0072$) (Table 2).

Relationship between VEGF or PD-ECGF expression and microvessel count

The microvessel counts in VEGF-positive pancreatic cancers (36.9 ± 3.1, mean ± standard error of mean) were significantly higher than those in VEGF-negative pancreatic cancers (21.4 ± 3.5) ($P = 0.002$) (Figure 3a). When the relationship between the expression of PD-ECGF staining and MVD was analysed in the strong and weak expression groups, the microvessel counts in the strong expression group (37.2 ± 4.2) were significantly higher than those in the weak expression group (26.4 ± 3.0) ($P = 0.044$) (Figure 3b). However, when the relationship between the expression of PD-ECGF staining and MVD were separately analysed for cancer cells and for infiltrating cells, there were no statistically significant differences (data not shown). As shown in Figure 3(c), the microvessel counts in patients with VEGF-positive and PD-ECGF-strong expression (double positive, $n = 14$) were also significantly higher than those in patients with VEGF-negative and PD-ECGF-weak expression (double negative, $n = 19$) ($P = 0.003$).

Relationship between VEGF or PD-ECGF expression and clinicopathological features, including survival analysis

Table 1 summarises the relationship between VEGF or PD-ECGF expression and clinicopathological features in 50 pancreatic cancers. Negative expression for VEGF and weak expression for PD-ECGF were significantly associated with well-differentiated adenocarcinomas. There was no statistical difference between these two angiogenic factors and other clinicopathological variables examined (Table 1).

The survival rates for 28 patients with VEGF-positive tumours and 22 with VEGF-negative tumours were not significantly different (Figure 2b). However, 17 patients with strong expression of PD-ECGF staining had a significantly worse survival than those with weak expression ($P = 0.011$) (Figure 2c). In addition, the survival rates for 14 patients with double positive expression (VEGF-positive and PD-ECGF-strong expression) and 19 patients with double negative expression (VEGF-negative and PD-ECGF-weak expression) were compared. No significant difference was found between the two groups, although patients with double negative expression tended to survive longer than those with double positive expression (Wilcoxon; $P = 0.09$).

RT-PCR analysis of VEGF mRNA isoforms and Western blot analysis of VEGF and PD-ECGF proteins

VEGF is encoded by a single-copy gene, that engenders multiple transcripts coding for the different protein isoforms

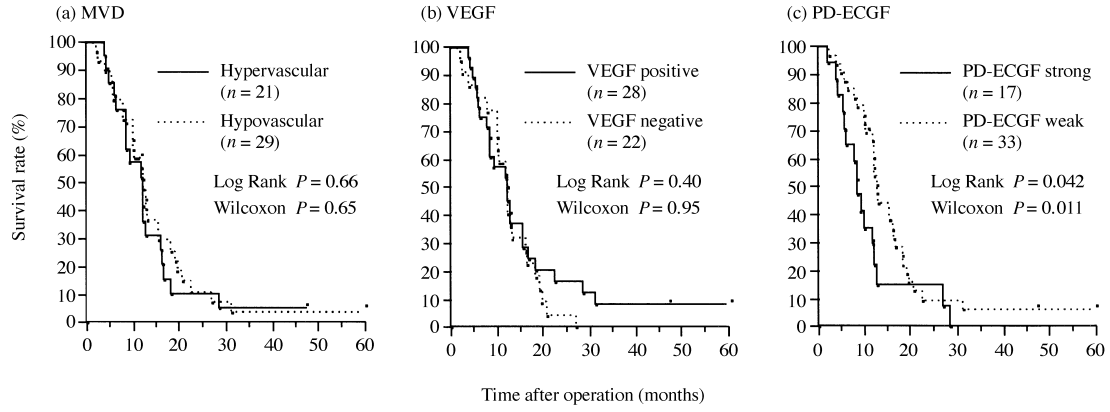

Figure 2. Kaplan–Meier survival curves with respect to (a) microvessel density (MVD); (b) vascular endothelial growth factor (VEGF) expression; and (c) platelet-derived endothelial cell growth factor (PD-ECGF) expression.

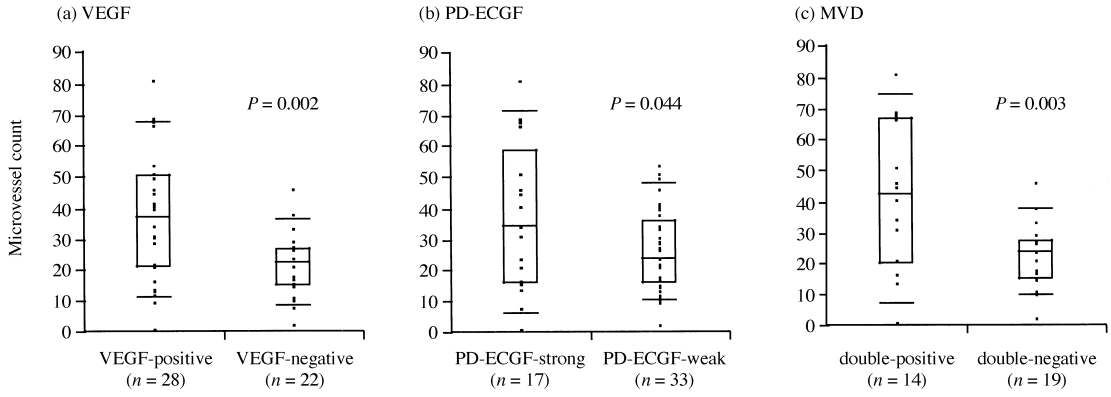

Figure 3. Correlation between (a) vascular endothelial growth factor (VEGF); (b) platelet-derived endothelial cell growth factor (PD-VEGF) expression; and (c) microvessel density (MVD). (c) Shows the microvessel counts in patients with VEGF-positive and PD-ECGF-strong expression (double positive) and those in patients with VEGF-negative and PD-ECGF-weak expression (double negative). In each figure, the box corresponds to the interquartile ranges, with the lower boundary of the box representing the 25th percentile and the upper boundary representing the 75th percentile. The line inside the box represents the mean value. The vertical line represents the 5th and 95th percentiles. *P* values were calculated by Student's *t* test.

$VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ [27]. To identify the mRNA species coding for the different VEGF isoforms produced by pancreatic cancer cell lines, RT–PCR analysis was performed. A representative experiment is shown in Figure 4. The predominant VEGF mRNA spliced forms detected were those coding for $VEGF_{121}$ and $VEGF_{165}$. The transcript coding for the higher molecular weight $VEGF_{189}$ isoform was solely detected in CFPAC-1.

Western blot experiments were performed on the total proteins extracted from five pancreatic cancer cell lines with anti-VEGF polyclonal and anti-PD-ECGF monoclonal antibodies to confirm the production of VEGF and PD-ECGF proteins. Western blot analysis of VEGF revealed two bands of 38 and 43 kDa in MIAPaCa-2 and CFPAC- 1, which were consistent with the reported molecular weights of $VEGF_{121}$ and $VEGF_{165}$ [28], and one band of 43 kDa was detected in three other cell lines (Figure 5a). Blots of PD-ECGF revealed the band of 55 kDa in four cancer cell lines, except for MIAPaCa-2, which is consistent with the reported molecular weight of PD-ECGF [24] (Figure 5b).

DISCUSSION

This study showed that human pancreatic cancers expressed VEGF and PD-ECGF, and that there was a statistically significant correlation between the expression of these angiogenic factors and the number of microvessels within the tumours.

The importance of angiogenesis in tumour development has been extensively described in recent years. Quantitation of intratumoral MVD has recently been shown to be a parameter of potential prognostic significance for various types of malignant tumours [2–6]. In this study, we evaluated the number of microvessels within a tumour by immunohistochemical analysis using anti-CD34 antibody as an endothelial cell marker. Several previous studies have revealed that

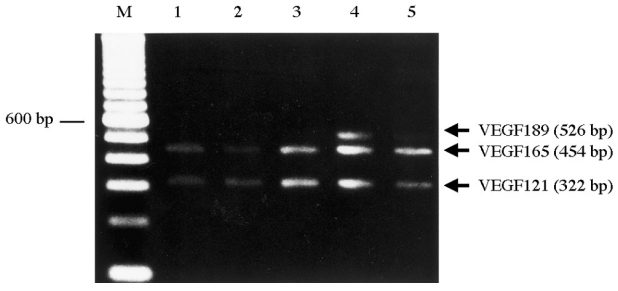

Figure 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of vascular endothelial growth factor (VEGF) in human pancreatic cancer cell lines. M, DNA molecular weight marker; lane 1, PANC-1; lane 2, AsPCA-1; lane 3, MIAPaCa-2; lane 4, CFPAC-1, lane 5, Capan-2 cell line.

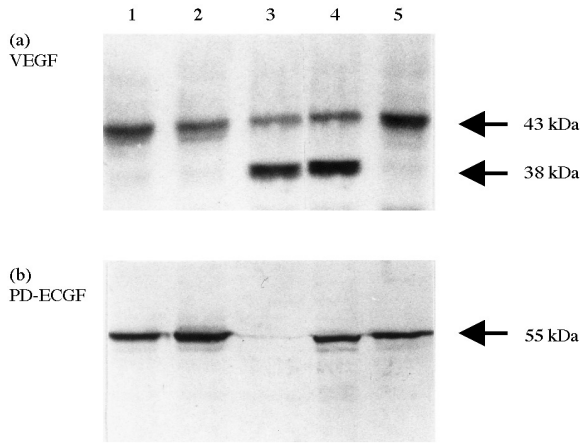

Figure 5. Western blot analysis of (a) vascular endothelial growth factor (VEGF); and (b) platelet-derived endothelial cell growth factor (PD-ECGF) proteins extracted from human pancreatic cancer cell lines. Fifty micrograms of total proteins extracted from cancer cells were loaded onto each lane. M, molecular weight marker; lane 1, PANC-1; lane 2, AsPC-1; lane 3, MIAPaCa-2; lane 4, CFPAC-1, lane 5, Capan-2 cell line.

anti-CD34 monoclonal antibody yielded consistent and reproducible staining of blood vessels in bladder, ovarian and gastric cancers and that microvessels stained with anti-CD34 antibody might provide more reliable information than those stained with von Willebrand's factor [3, 4, 29]. We found that the mean vessel count per high-power field in pancreatic cancer specimens from the entire group of 50 patients was 30.1. This was significantly lower than the mean vessel count of approximately 80 in bladder cancers [3] and 144 in gastric carcinomas [4] determined in the same manner using the same antibody. This result appears to be consistent with the clinical finding on dynamic CT and angiography that pancreatic cancer is a hypovascular tumour [21]. It has been demonstrated that the microvessel count is a significant predictor of recurrence and survival in various malignancies [2–6]. However, recent publications have shown that MVD is not a reliable predictor of metastasis-free survival or overall survival in colon cancer [30, 31]. In the present study, we found that advanced stage of disease was more frequent in the hypervascular group, but could not show any relationships between MVD and pT, lymph node metastasis and hepatic metastasis. Furthermore, the survival rates for the hypervascular and the hypovascular groups were not significantly different. These results may suggest that MVD is not useful for predicting either the risk of metastasis in pancreatic cancers or the prognosis of the patients.

Considerable attention has been paid to the mechanisms of tumour angiogenesis in recent years. Most work has been directed toward the identification of the various peptide growth factors that stimulate proliferation and motility of endothelial cells to induce new blood vessel formation. Interestingly, several investigators have shown that the existence of hypoxic conditions within tumours increases the gene expression of a variety of proteins, including the angiogenic factors VEGF and PD-ECGF in tumour cells [16, 17]. In the present study, we focused on the expression of VEGF and PD-ECGF among a variety of angiogenic factors, since pancreatic cancer is composed of abundant fibrotic components and has been considered to be more hypoxic than other malignancies.

VEGF is a 38–46 kDa secreted, dimeric N-glycoprotein which is chemotactic and mitogenic for endothelial cells *in vitro* [7], elicits angiogenesis *in vivo*, and increases the permeability of the vascular endothelium [32]. Several recent studies have shown that VEGF may be a potential mediator of tumour-associated neoangiogenesis *in vivo* since it is upregulated in various human tumours [2, 8]. We examined the mRNA and protein expressions of VEGF in five human pancreatic cancer cell lines by using RT–PCR and/or Western blot analysis. In humans, four different VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) have been identified, and it has been shown that $VEGF_{121}$ and $VEGF_{165}$ have different biochemical and biological properties from those of $VEGF_{189}$ and $VEGF_{206}$. $VEGF_{121}$ and $VEGF_{165}$ are soluble proteins, whereas $VEGF_{189}$ and $VEGF_{206}$ are mostly cell-associated, since these isoforms contain basic, heparin-binding peptides [8, 33]. In this study, two smaller forms of VEGF transcripts and protein isoforms ($VEGF_{121}$ and $VEGF_{165}$) were identified, and were thought to be the predominant species produced in pancreatic cancer cells (Figures 4, 5a). VEGF immunostaining was found to be positive in 28 of 50 pancreatic cancers (56%), and a statistically significant correlation was found between VEGF expression and the microvessel counts ($P = 0.002$) (Figure 3a). These findings might suggest that $VEGF_{121}$ and $VEGF_{165}$ produced by and secreted from pancreatic cancer cells activate the corresponding cognate receptors on endothelial cells, thus establishing a paracrine mechanism, which could ultimately lead to the neoformation of blood vessels.

PD-ECGF is a 55 kDa polypeptide existing *in vivo* as a 110 kDa homodimer and an endothelial cell mitogen initially purified to homogeneity from human platelets [34]. PD-ECGF has been demonstrated to induce chemotaxis of endothelial cells and angiogenesis *in vivo* [34]. The amino acid sequence for PD-ECGF has been found to be identical to that of thymidine phosphorylase, an enzyme involved in pyrimidine nucleoside metabolism [35]. Haraguchi and colleagues indicated that PD-ECGF stimulates the chemotaxis of endothelial cells through the degradation products of thymidine, and, thus, indirectly induces angiogenesis [36, 37]. Several authors have recently shown that PD-ECGF, as well as VEGF, is an important molecule in human tumour angiogenesis [9, 10, 12]. In this study, we used Western blot analysis to examine the protein expression of PD-ECGF in pancreatic cancer cell lines and detected a single protein band of 55 kDa in four pancreatic cancer cell lysates, except for MIAPaCa-2 (Figure 5b). In the immunohistochemical analysis of the resected specimens of pancreatic cancers, PD-ECGF overexpression was found in the cytoplasms and/or nuclei of cancer cells (44%) and the infiltrating cells (50%) that might be either macrophages or fibroblasts. Takebayashi and associates demonstrated that higher levels of PD-ECGF expression in cancer cells in colorectal carcinomas were associated with more extensive angiogenesis and unfavourable clinical outcome, but their data showed that the staining of cancer cells was not especially strong [9]. Furthermore, Takahashi and colleagues demonstrated that colon cancer cells rarely stained for PD-ECGF; instead, most staining occurred in the infiltrating cells, and PD-ECGF mRNA was not detected in a pure population of all of four human colon cancer cell lines, indicating that infiltrating cells expressing PD-ECGF might be important and contribute to angiogenesis [10]. Our data were slightly different from theirs, since

we detected the expression of PD-ECGF in four of five human pancreatic cancer cells by using Western blot analysis. We consider the expression of PD-ECGF in cancer cells and infiltrating cells to be equally important for angiogenesis in pancreatic cancers. Therefore, the positivity (weak or strong) of PD-ECGF staining was estimated by summing the intensities of both cells, and we found that a strong expression of PD-ECGF significantly correlated with intratumoral microvessel counts ($P = 0.044$).

Various studies have shown that VEGF or PD-ECGF expression is significantly associated with advanced stage, high incidence of distant metastases after surgery, and less favourable prognosis in a number of malignancies [2, 9, 12, 38, 39]. Furthermore, some investigators have recently demonstrated that VEGF stimulates mitogenesis in adult rat pancreatic ductal epithelium *in vitro* and *in vivo* [40, 41]. In this study, the expression of VEGF and PD-ECGF did not correlate with clinicopathological features, except for tumour histology. VEGF expression was not a significant prognostic indicator. However, patients with a strong PD-ECGF expression had a significantly less favourable survival than those with a weak expression ($P = 0.011$).

Takahashi and colleagues demonstrated that the intensity of staining for PD-ECGF did not correlate with the intensity of VEGF staining in colon cancer [10], while other investigators demonstrated that VEGF and PD-ECGF were frequently coexpressed in breast cancer specimens [15]. Our study indicated a highly significant correlation between VEGF and PD-ECGF expression ($P = 0.0053$), and the microvessel counts in tumours with VEGF-positive and PD-ECGF-strong expression (double positive) were also significantly higher than in those with VEGF-negative and PD-ECGF-weak expression (double negative) ($P = 0.003$). Thus, angiogenesis in pancreatic cancer may involve a co-ordinate regulation of these two angiogenic factors.

In conclusion, this study has demonstrated that mRNA and protein of VEGF and PD-ECGF are expressed in pancreatic cancer cells and/or infiltrating cells, and that the overexpression of these angiogenic factors significantly stimulates tumour angiogenesis, as assayed by intratumoral MVD. Furthermore, the expression of PD-ECGF correlated with poor survival of the patients, but MVD and VEGF expression were not found to be useful for the prediction of overall survival. This study, therefore, suggests that VEGF and PD-ECGF may play an important role in tumour angiogenesis, and that PD-ECGF expression seems to be useful for establishing prognoses for pancreatic cancer.

1. Folkman J. What is the evidence that tumors are angiogenesis dependent? [editorial]. *J Natl Cancer Inst* 1990, **82**, 4–6.
2. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995, **55**, 3964–3968.
3. Bochner BH, Cote RJ, Weidner N, *et al.* Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. *J Natl Cancer Inst* 1995, **87**, 1603-1612.
4. Tanigawa N, Amaya H, Matsumura M, *et al.* Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. *Cancer Res* 1996, **56**, 2671–2676.
5. Gasparini G. Angiogenesis research up to 1996. A commentary on the state of art and suggestions for future studies. *Eur J Cancer* 1996, **32A**(14), 2379–2385.
6. Gasparini G. Clinical significance of the determination of angiogenesis in human breast cancer: update of the biological background and overview of the Vicenza studies. *Eur J Cancer* 1996, **32A**(14), 2485–2493.
7. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 1989, **161**, 851–858.
8. Viglietto G, Romano A, Maglione D, *et al.* Neovascularization in human germ cell tumors correlates with a marked increase in the expression of the vascular endothelial growth factor but not the placenta-derived growth factor. *Oncogene* 1996, **13**, 577–587.
9. Takebayashi Y, Akiyama S, Akiba S, *et al.* Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phophorylase, in human colorectal carcinoma. *J Natl Cancer Inst* 1996, **88**, 1110–1117.
10. Takahashi Y, Bucana CD, Liu W, *et al.* Platelet-derived endothelial cell growth factor in human cancer angiogenesis: role of infiltrating cells. *J Natl Cancer Inst* 1996, **88**, 1146–1151.
11. Mise M, Arii S, Higashituji H, *et al.* Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 1996, **23**, 455–464.
12. Koukourakis MI, Giatromanolaki A, O'Byrne M, *et al.* Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small-cell lung cancer. *Br J Cancer* 1997, **75**, 477–481.
13. Rosenberg JM, Welch JP, Macaulay WP. Cancer of the head of the pancreas. *J Surg Oncol* 1985, **28**, 217–221.
14. Egawa S, Tsutsumi M, Konishi Y, *et al.* The role of angiogenesis in the tumor growth of Syrian hamster pancreatic cancer cell line HPD-NR. *Gastroenterology* 1995, **108**, 1526–1533.
15. Toi M, Inada K, Hoshina S, Suzuki H, Konda S, Tominaga T. Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res* 1995, **1**, 961–964.
16. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992, **359**, 843–845.
17. Griffiths L, Dachs GU, Bicknell R, Harris AL, Stratford IJ. The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells grown *in vitro* and *in vivo*. *Cancer Res* 1997, **57**, 570–572.
18. Larcher F, Robles Al, Duran H, *et al.* Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels. *Cancer Res* 1996, **56**, 5391–5396.
19. Enholm B, Paavonen K, Ristimaki A, *et al.* Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 1997, **14**, 2475–2483.
20. White FC, Benehacene A, Scheele JS, Kamps M. VEGF mRNA is stabilized by ras and tyrosine kinase oncogenes, as well as by UV radiation–evidence for divergent stabilization pathways. *Growth Factors* 1997, **14**, 199–212.
21. Hosoki T. Dynamic CT of pancreatic tumors. *AJR* 1983, **140**, 959–965.
22. Gansauge S, Gansauge F, Beger HG. Molecular oncology in pancreatic cancer. *J Mol Med* 1996, **74**, 313–320.
23. Sobin L, Wittekind C, eds. *UICC TNM Classification of Malignant Tumours*. Berlin, Springer, 1997.
24. Takebayashi Y, Yamada K, Miyadera K, *et al.* The activity and expression of thymidine phosphorylase in human solid tumors. *Eur J Cancer* 1996, **32A**(7), 1227–1232.
25. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Eng J Med* 1991, **324**, 1–8.
26. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Biochem* 1976, **72**, 248–254.
27. Tisher E, Mitchell R, Hartman T, *et al.* The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991, **266**, 11947–11954.

28. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrine Rev* 1992, **13**, 18–32.
29. Hollingsworth HC, Kohn EC, Steinberg SM, Rothenberg ML, Merino MJ. Tumor angiogenesis in advanced stage ovarian carcinoma. *Am J Pathol* 1995, **147**, 33–41.
30. Bossi P, Viale G, Lee AK, Alfano R, Coggi G, Bosari S. Angiogenesis in colorectal tumors: microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. *Cancer Res* 1995, **55**, 5049–5053.
31. Mooteri S, Rubin D, Leurgans S, Jakate S, Drab E, Saclarides T. Tumor angiogenesis in primary and metastatic colorectal cancers. *Dis Colon Rectum* 1996, **39**, 1073–1080.
32. Connolly DT, Heuvelman DM, Nelson R, *et al.* Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989, **84**, 1470–1478.
33. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989, **246**, 1306–1309.
34. Ishikawa F, Miyazono K, Hellman U, Drexler H, Wernstedt C, Hagiwara K. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature* 1989, **338**, 557–562.
35. Furukawa T, Yoshimura A, Sumizawa T, Haraguchi M, Akiyama S, Fukui K. Angiogenic factor [letter]. *Nature* 1992, **356**, 668.
36. Haraguchi M, Miyadera K, Uemura K, *et al.* Angiogenic activity of enzymes. *Nature* (London) 1994, **368**, 198.
37. Folkman J. What is the role of thymidine phosphorylase in tumor angiogenesis? [Editorial]. *Natl Cancer Inst J* 1996, **88**, 1091–1092.
38. Relf M, LeJeune S, Scott PAE, *et al.* Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor B-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Res* 1997, **57**, 963–969.
39. Inoue K, Ozeki Y, Suganuma T, Sugiura Y, Tanaka S. Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma. Association with angiogenesis and tumor progression. *Cancer* 1997, **79**, 206–213.
40. Oberg-Welsh C, Sandler S, Anderson A, Welsh M. Effects of VEGF in pancreatic duct cell replication and the insulin production of fetal islet-like cell clusters in vivo. *Mol Cell Endocrinol* 1997, **126**, 125–132.
41. Rooman I, Schuit F, Bouwens L. Effects of VEGF on growth and differentiation of pancreatic ductal epithelium. *Lab Invest* 1997, **76**, 225–232.

Acknowledgement—This study was supported in part by a grant from the Ministry of Education of Japan.