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Pilot study to relate clinical outcome in pancreatic carcinoma and angiogenic plasma factors/circulating mature/progenitor endothelial cells: Preliminary results

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Circulating endothelial cells (CEC) and bone marrow-derived endothelial progenitors (ECP) play important roles in tumor growth and have been proposed as non-invasive markers of angiogenesis. However, CEC and ECP levels have not been investigated in pancreatic carcinoma patients. Using four-color flow cytometry procedures, we evaluated the count of resting (rCEC) and activated (aCEC) endothelial cells and ECP in the peripheral blood of pancreatic carcinoma patients before and after chemotherapy, consisting of gemcitabine (GEM) alone or in combination with oxaliplatin (OX), or with 5-fluorouracil (5-FU). We also correlated CEC and ECP levels with plasma levels of relevant angiogenic factors, such as vascular endothelial growth factor (VEGF)-A, VEGF-D, angiopoietin (Angio)-1, and chemokine C-X-C motif ligand (CXCL)12, measured by ELISA, and with clinical features of pancreatic cancer. The aCEC, rCEC, ECP, and VEGF-A plasma levels were significantly higher in locally-advanced and metastatic patients than controls. Both ECP and VEGF-A levels correlated positively with disease stage and inversely with patient's overall survival. Measurements after the treatment course showed that VEGF-A plasma concentrations and ECP counts had decreased significantly. In particular, VEGF-A and rCEC were significantly down after treatment with GEM alone or in combination with OX. No significant differences in terms of circulating angiogenic factor or endothelial cell subtype levels were found between responders (patients entering partial remission or with stable disease) and non-responders (patients with progressive disease). The study provides insights into angiogenesis mechanisms in pancreatic carcinoma, for which anti-angiogenic targeting of VEGF-A and ECP could be of interest. (*Cancer Sci* 2010; 101: 2448–2454)

Pancreatic carcinoma is a biologically-aggressive malignancy with a propensity to spread locally and metastasize distally. Although not grossly vascular, these cancers exhibit foci of micro-angiogenesis and overexpress the paramount pro-angiogenic factor vascular endothelial growth factor (VEGF), and also additional mitogenic growth factors that are likewise angiogenic.[1–4]

Most tumors form vessels through sprouting or co-option of neighboring pre-existing vessels.[5] However, there is mounting evidence that an adapted form of the embryonic process of vasculogenesis might contribute to tumor growth and spread. In this process, bone marrow-derived hemopoietic stem cells and endothelial progenitors, mobilized by tumor- and/or ischemia-induced signals, home onto the tumor site and contribute to the formation of new vessels.[6] In animal models, both angiogenic processes are essential for tumor growth.[7]

Several assays have recently been developed to detect and quantify circulating endothelial cells (CEC), which comprise mature (r)CEC derived from the vessel wall, cytokine-activated (a) CEC, and endothelial cell progenitors (ECP) recruited from the bone marrow.[8–10]

As an alternative to, or in association with, the standard of care for the treatment of pancreatic carcinoma patients, anti-angiogenic and vascular-disrupting agents are currently under investigation as novel approaches to the management of this cancer, which is resistant to the routine use of chemotherapy.[11] Increasing evidence in several hematological[12–16] and non-hematological malignancies[17–27] indicates that CEC and ECP may be non-invasive markers of angiogenesis, in addition to classical biomarkers such as circulating angiogenic factors.[28] The levels of CEC and ECP in patients with pancreatic carcinoma have not been investigated to date.

The study aimed to analyze the presence of rCEC, aCEC, and ECP and the levels of angiogenesis-related proteins, that is, VEGF-A, VEGF-D, angiopoietin (Angio)-1, and C-X-C motif ligand (CXCL) in the blood of patients with locally advanced or metastatic pancreatic carcinoma, before and after different chemotherapy regimens.

Materials and Methods

Patients. The series comprised 34 patients diagnosed with advanced or metastatic pancreatic adenocarcinoma at the Department of Clinical Oncology, San Giovanni Battista Hospital (Turin, Italy), between September 2001 and January 2006. Using the International Union Against Cancer (UICC) Staging System,[29] patients were staged as: IIa (R1) (n = 3); IIb (R1) (n = 2); III (n = 9); and IV (n = 20) at time of eligibility for chemotherapy. Fifteen patients underwent surgery: seven had radical surgery (R0), and were submitted to chemotherapy only at disease progression; five patients with microscopic residual (R1); and three received palliative surgery and underwent chemotherapy immediately afterwards. Patients' clinico-pathological features are listed in Table 1. None had undergone anticancer treatment before entering the study. All participating patients gave informed consent before entering the study and the procedures followed were in accordance with the Helsinki Declaration. As first-line chemotherapy, 21 patients received gemcitabine (GEM) (1250 mg/m^2 at days 1 and 8, every 21 days), nine received GEM (1000 mg/m^2 at day 1) plus oxaliplatin (OX) (100 mg/m^2 at day 2, every 14 days), three received

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Table 1. Clinico-pathological features of patients with pancreatic carcinoma who participated in this study (n = 34)

Characteristic		Number	
Gender (male/female)		19/15	
Median age		63 years (range, 47–76)	
Disease stage at beginning of chemotherapy	R1 after surgery	5	
	Stage III	9	
	Stage IV	20	
Surgery	Yes	15	→ 7 R0 → 5 R1 → 3 palliative
	No	19	
Metastasis site	Liver	11	
	Liver + peritoneum	3	
	Lung	2	
	Peritoneum	2	
	Liver + adrenal gland	1	
	Lymph node	1	
Chemotherapy regimens	GEM	21	
	GEMOX	9	
	GEM + 5-FU	3	
	OX + 5-FU	1	
Radiological response rate	RC	0	
	RP	5	
	SD	12	
	PD	17	

5-FU, 5-fluorouracil; GEM, gemcitabine; OX, oxaliplatin; PD, progressive disease; R0, no evidence of disease; R1, microscopic residual disease; RC, complete remission; RP, partial remission; SD, stable disease.

GEM + 5-fluorouracil (5-FU) (GEM 1000 mg/m^2 on days 1, 8, and 15, every 28 days; 5-FU 200 mg/m^2/day in continuous infusion for 7 days, from days 1 to 21), and one received OX (40 mg/m^2) + 5-FU (500 mg/m^2) as a bolus and leucovorin (250 mg/m^2) on days 1, 8, and 15, on a 28 day cycle. All patients were observed until they died or until April 2009. At that time, only one patient was surviving, after radical surgery and 4 months of chemotherapy. All other patients (n = 33) had died of the disease.

Determination of VEGF-A, VEGF-D, Angio-1, and CXCL12 in plasma. Plasma samples with EDTA were collected from patients on the day of admission (time 0) (before chemotherapy) and at the first restaging (after an interval of 2–3 months, depending on the chemotherapy regime) in parallel with evaluation of the clinical course, and from age- and sex-matched hospital staff volunteers (n = 26) as control. We used ELISA to determine VEGF-A, VEGF-D, Angio-1, and CXCL12 using commercially available kits from Bender MedSystems (Burlingame, CA, USA), R&D Systems (Abingdon, UK), and RayBiotech (Norcross, GA, USA). All samples were evaluated in duplicate. The minimum detectable doses were below 7.9, 11.4, 30, and 18 pg/mL, respectively.

Flow cytometry analysis. Patients' blood samples were collected at the time of clinical assessment in tubes containing EDTA. We enumerated CEC, aCEC, and ECP by four-color, rare event, flow cytometry analysis, following the procedure of Mancuso *et al.*,[29] using optimized concentrations of a panel of mAbs, including peridinin chlorophyll protein (PerCP)-conjugated anti-CD45 (BD Pharmingen, San Diego, CA, USA) to exclude hematopoietic cells, FITC-conjugated anti-CD31 (BD Pharmingen) and anti-CD146 (Chemicon, Temecula, CA, USA), phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD105 (endoglin) (Euroclone, Devon, UK), and PE Texas Red (ECD)-conjugated anti-CD34 (Beckman-Coulter, High Wycombe, UK).

Fluorochrome and isotype matched controls, as well as unstained cell samples, were measured and processed as negative controls to set the appropriate regions. Cell viability was assessed by DNA intercalator propidium iodide. The gating strategy described in detail elsewhere[11,15,31] was applied to identify CEC and ECP subtypes, excluding interfering red blood cells, platelets, dead cells, cell debris, and neutrophils and the reference fluorescent beads (Flow Count beads; Beckman-Coulter, Fullerton, CA, USA) used to obtain absolute cell count, subsequently excluding hematopoietic cells expressing the CD45 antigen. Endothelial progenitors were defined as negative for hematopoietic marker CD45 and positive for endothelial cell markers CD34, CD31, and the ECP marker CD133. Mature rCEC were defined as CD45$^-$, CD133$^-$, CD105$^-$, CD31$^+$, CD34$^+$, and CD146$^+$. Activated CEC were classified as CD105$^+$ mature CEC. Absolute cell numbers were calculated with reference to fluorescent beads by the so-called ''single platform method''.[32] A direct lyse-no-wash procedure was used to avoid cell or bead loss. Each sample was analyzed for a minimum of 100 000 total events on a Coulter Epics XL flow cytometer (Beckman-Coulter). Data were analyzed in duplicate by the same investigator using Expo 32 software (Beckman-Coulter). The absolute CEC and CEP numbers (cell/μL) were calculated from the following formula: number of measured CEC or CEP/number of fluorescent beads counted × number of beads/μL.

Statistical analysis. Variables were compared by means of the Student's t-test or the non-parametric Mann–Whitney U-test for intergroup comparisons. Result correlation was calculated with the Spearman's rank correlation test. Overall survival rates were estimated by the Kaplan–Meier method. Significance between survival curves was assessed with the log–rank test. The Cox proportional hazard model was applied to identify prognostic factors. Hazard ratios, along with 95% CI, were provided. P-values below 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS software (13.0) for Windows (SPSS, Chicago, IL, USA).

Results

Plasma levels of VEGF-A, VEGF-D, Angio-1, and CXCL12. To better define the systemic characterization of the angiogenic profile of pancreatic carcinoma, VEGF-A, VEGF-D, Angio-1, and CXCL12 plasma concentrations were measured by ELISA in patients with locally advanced or metastatic pancreatic carcinoma before chemotherapy, and compared with the corresponding levels of healthy donors. As shown in Figure 1(a), there were no statistically significant differences in levels of CXCL12 or Angio-1 between normal donors (n = 26) and patients (n = 34) [median (range): 3719.9 (153.6–6451.1) pg/mL *vs* 3892.7 (3686.4–4780.7), P = 0.080; 6060.8 (2254.1–9016.6) pg/mL *vs* 4904.2 (1813.1–8304.6) pg/mL, P = 0.201, respectively)]. In contrast, patients had significantly higher median plasma levels of VEGF-A *vs* healthy donors [median (range): 539.1 (136.2–1892.4) pg/mL *vs* 105.7 (321–59.3) pg/mL, $P < 0.001$)]. Interestingly, plasma levels of VEGF-D were lower in patients than in controls [median (range), 174.1 (12.1–781) pg/mL *vs* 396.6 (61.5–856.2) pg/mL, P = 0.036]. No significant correlation emerged between circulating levels of VEGF-A, VEGF-D, CXCL12, or Angio-1, either in pancreatic carcinoma patients or in controls (Spearman's correlation test, $P > 0.05$).

Circulating endothelial cell subtype counts. As angiogenic factors are involved in the recruitment and mobilization of mature endothelial cells and their progenitors, in 15 of 34 patients we also investigated the concentrations of circulating endothelial cell types. The different subsets rCEC, aCEC, and

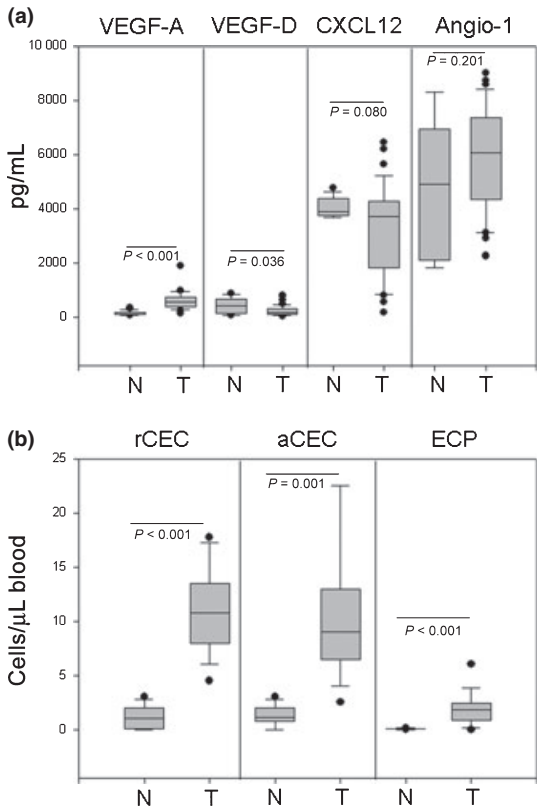

Fig. 1. Basal vascular endothelial growth factor (VEGF)-A, VEGF-D, chemokine C-X-C motif ligand (CXCL) 12 and angiopoietin (Angio)-1 levels in plasma, and resting circulating endothelial cells (rCEC), activated circulating endothelial cells (aCEC), and endothelial progenitor cells (ECP) counts in the peripheral blood of patients with locally advanced or metastatic pancreatic carcinoma (T), and of normal donors (N). (a) Plasma levels of angiogenic factors were determined by ELISA. (b) Both CEC and ECP numbers were determined by flow cytometry. Median, 10th, 25th, 75th, and 90th percentiles are presented as vertical boxes with error bars. Dots indicate outliers. *P*-values obtained by the Mann–Whitney *U*-test.

ECP were defined on the basis of their distinct phenotypic characteristics and accurate sequential gating by flow cytometry analysis, as described above. As shown in Figure 1(b), in pancreatic carcinoma patients ($n = 15$), the number of rCEC, aCEC, and ECP were 10-, 8-, and 26-fold, respectively, those of controls ($n = 15$) [median (range): rCEC, 10.7 (4.5–17.7)/μL *vs* 1 (0–3)/μL, $P < 0.001$; aCEC, 9 (2.5–22.5)/μL *vs* 1.1 (0–3)/μL, $P < 0.001$; ECP, 1.8 (0.0–6.0)/μL *vs* 0.07 (0.00–0.14)/μL, $P < 0.001$]. No correlations were found between rCEC, aCEC, or ECP, either in pancreatic carcinoma patients or in controls ($P > 0.05$).

Correlation between rCEC, aCEC, and ECP number and VEGF-A, VEGF-D, CXCL12, and Angio-1 plasma levels. To investigate whether the presence of increased numbers of CEC and ECP in patients with locally advanced or metastatic pancreatic carcinoma depends on the plasma levels of angiogenic factors, Spearman's rank correlation test was applied to the amounts of rCEC, aCEC, and ECP detected by flow cytometry and to the plasma concentrations of the angiogenic factors. Subsets of CEC and ECP were not significantly associated with VEGF-D, CXCL12, or Angio-1 ($P > 0.05$). A positive correlation with VEGF-A plasma levels was found only for ECP ($R = 0.707$, $P = 0.003$).

Correlation of circulating angiogenic factor levels, CEC subset, and ECP numbers with disease stage and overall survival. To clarify the clinical significance of VEGF-A, VEGF-D, CXCL12, Angio-1, rCEC, aCEC, and ECP in locally advanced or metastatic pancreatic carcinoma, the correlation was examined between disease stage at time of eligibility for chemotherapy, and circulating levels of the four factors and CEC particular subtypes and ECP, measured before the start of treatment.

When plasma levels of the angiogenic factors were correlated with patients' TNM stages ($n = 34$, of whom 4 were stage II, 9 were stage III, and 21 were stage IV), a statistically significant correlation was found only between disease stage and VEGF-A ($R = 0.476$, $P = 0.005$). When rCEC, aCEC, and ECP numbers were correlated with patients' TNM stages ($n = 15$, of whom 2 were stage II, 3 were stage III, and 10 were stage IV), a statistically significant correlation was only observed for ECP ($R = 0.543$, $P = 0.03$).

Additionally, Kaplan–Meier curves for overall patient survival, using median angiogenic factor plasma levels and rCEC, aCEC, and ECP amounts as cut-off points between low and high level patient groups, combined with univariate analysis, showed that there was no difference in overall survival of the two groups, in the case of VEGF-D, CXCL12, Angio-1, rCEC, and aCEC. However, patients with high VEGF-A plasma levels (≥539.1 pg/mL) and ECP counts (≥10.7 cells/μL) had significantly shorter overall survival ($P = 0.050$ and $P = 0.030$, respectively) (Fig. 2).

Logistic regression analysis showed ECP counts to be significantly associated with increased risk of poor prognosis [Odds ratio (OR), 4.49; 95% CI, 1.60–12.59; $P = 0.004$), and a trend toward this association was found for VEGF-A (OR, 6.50; 95% CI, 0.88–47.90; $P = 0.058$).

Effect of different chemotherapy regimens on VEGF-A, VEGF-D, CXCL12, Angio-1, rCEC, aCEC, and ECP levels. Patients were treated with one of four different combination regimens (GEM, $n = 21$; GEMOX, $n = 9$; GEM + 5-FU, $n = 3$; OX + 5-FU and leucovorin, $n = 1$). The three patients receiving GEM + 5-FU and the single patient receiving OX + 5-FU and leucovorin were not included in this analysis.

When the patients were categorized by chemotherapy regimen, as shown in Table 2, those receiving either of the GEM treatments showed no significant difference with regard to pre- and post-plasma levels of VEGF-D, CXCL12, or Angio-1. In contrast, there was a statistically significant decrease in VEGF-A levels ($P < 0.001$). When GEM was associated with OX, VEGF-A levels significantly decreased ($P = 0.004$), while VEGF-D levels increased ($P = 0.045$), reaching near-normal levels. The levels of CXCL12 and Angio-1 remained unchanged. Moreover, of the 15 patients who were also evaluated for rCEC, aCEC, and ECP counts, eight were treated with GEM and five with GEMOX. The other two patients, receiving OX + 5-FU and leucovorin, and GEM + 5-FU, respectively, were not considered. As shown in Table 2, significant differences were found in rCEC counts before and after GEM monotherapy or GEMOX treatment ($P = 0.025$ and $P = 0.028$, respectively). Even considering only this small subgroup of patients, treatment with GEM alone or in combination with OX, induces a significant reduction in VEGF-A levels ($P = 0.045$ and $P = 0.034$, respectively).

Levels of VEGF-A, VEGF-D, CXCL12, Angio-1, rCEC, aCEC, and ECP and objective tumor response to chemotherapy. The overall chemotherapy response rate was 50% (17 of 34 patients, of whom five went into partial remission and 12 had stable disease). When basal VEGF-A, VEGF-D, CXCL12, and Angio-1 plasma levels and rCEC, aCEC, and ECP counts of patients who had disease progression (classified as non-responders) were compared with those of patients who went into partial remission or had stable disease (classified as responders), no statistically significant differences were found ($P > 0.050$).

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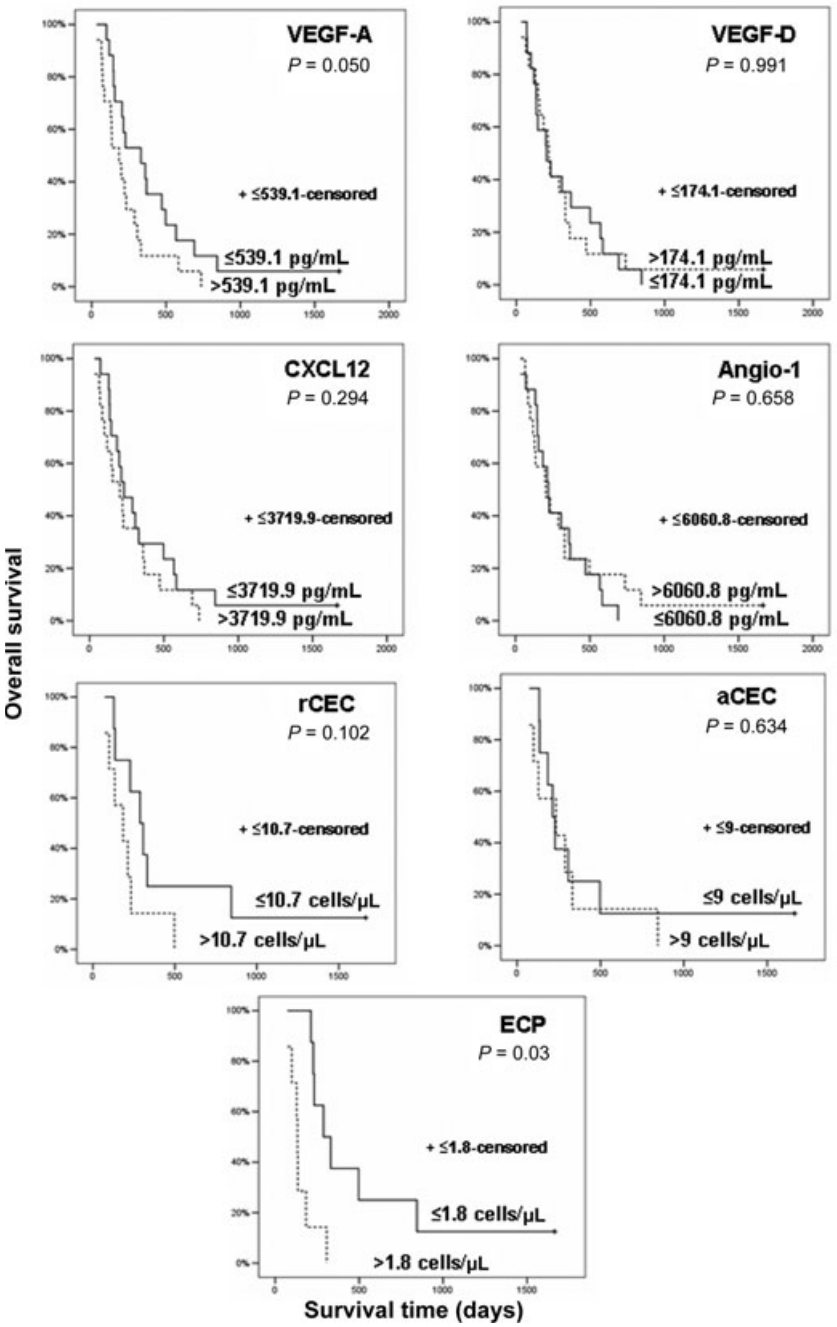

Fig. 2. Overall survival (analyzed by the Kaplan–Meier method) for patients with high and with low vascular endothelial growth factor (VEGF)-A, VEGF-D, chemokine C-X-C motif ligand (CXCL) 12, and angiopoietin (Angio)-1 plasma levels, and resting circulating endothelial cells (rCEC), activated circulating endothelial cells (aCEC), and endothelial progenitor cells (ECP) numbers. Median angiogenic factor plasma levels and rCEC, aCEC, and ECP amounts was used as cut-off points. Both VEGF-A and ECP were significantly and inversely associated with patient survival (log–rank test).

Discussion

Although an optimal enumeration technique and consensus have not yet been achieved on the phenotypic definition of CEC and ECP, elevated numbers of CEC have been reported in several tumor types, indicating a perturbation of the vascular endothelium in cancer disease. On the basis of these observations, CEC and ECP measurements have attractive potential prognostic and therapeutic applications for malignant diseases.[14–16,33–37] Pancreatic carcinoma is usually diagnosed at an advanced stage, which in part explains its high resistance to chemotherapy and radiotherapy. A better understanding of the mechanisms that contribute to angiogenesis and metastasis in this highly lethal cancer could greatly enhance the efficacy of novel curative strategies and improve patients' long-term survival.

To date, no studies have addressed CEC and ECP in pancreatic carcinoma patients. This pilot study analyzed the levels of these cells and of relevant circulating angiogenic factors in patients with locally advanced or metastatic pancreatic carcinoma, before and after palliative chemotherapy with GEM alone or GEM associated with OX, by four-color flow cytometry for CEC and ECP, and by ELISA for angiogenic proteins. Results were compared with those of matched normal subjects, and correlations with clinical features were also investigated.

Circulating endothelial cell levels were approximately 10 times higher in locally advanced or metastatic pancreatic carcinoma patients compared to healthy controls, and ECP even more so (26 times). This is consistent with findings of other studies that have reported increased levels of CEC and ECP in patients with various cancer types.[5,10,11,14–16,34,38,39]

Table 2. Effect of different chemotherapeutic regimens on resting circulating endothelial cell (rCEC), activated circulating endothelial cell (aCEC), and endothelial progenitor cell (ECP) counts in patients with pancreatic carcinoma

Chemotherapy regimen	Number/μL, median (range)				Plasma levels (pg/mL), median (range)		
	rCEC	aCEC	ECP	VEGF-A	VEGF-D	CXCL12	Angio-1
Time 0†	10.7 (7.7–17.0)	8.2 (2.5–22.5)	1.9 (0.0–2.4)	495.3 (136.2–786.4)	181.6 (12.1–781.0)	1118.4 (153.6–6451.1)	6548.8 (2254.1–9016.6)
GEM (n = 8)‡	6.7 (3.6–9.0)	7.0 (3.3–12.0)	0.7 (0.1–3.0)	344.4 (163.0–508.9)	69.6 (0.0–1495.5)	1502.4 (1238.4–2332.8)	6514.1 (3442.0–7728.0)
P*	0.025	0.384	0.668	0.045	0.945	0.835	0.712
Time 0	13.5 (7.0–17.7)	7.5 (6.0–15.5)	1.8 (0.3–6.0)	703.2 (532.1–1892.4)	175.6 (112.0–336.0)	2150.4 (1065.6–4751.9)	6116.4 (2917.5–8731.8)
GEM + OX (n = 5)	8.2 (5.6–13.8)	7.6 (2.0–18.0)	2.5 (0.4–5.7)	456 (377.3–1234.0)	387.5 (196.8–1104.9)	2476.8 (278.3–3062.4)	3959.5 (1431.0–6675.6)
P	0.028	0.930	0.664	0.034	0.179	0.494	0.981

*P-values by Wilcoxon signed rank test. †Before chemotherapy. ‡First restaging after chemotherapy. Angio-1, angiopoietin-1; CXCL12, chemokine C-X-C motif ligand 12; GEM, gemcitabine; OX, oxaliplatin; VEGF, vascular endothelial growth factor.

Possible mechanisms underlying the increase in numbers of these cell types in pancreatic carcinoma and in other malignancies may include a generalized activation of the endothelium, localized endothelial damage by the tumor itself, or an elevation of precursor cells. The finding that both rCEC that aCEC are elevated in pancreatic carcinoma patients suggests that these cells derive either from angiogenic microvessels or from distant, uninvolved vessels activated by angiogenic cytokines. In contrast, ECP, which possess the ability to migrate, colonize, proliferate and, ultimately, differentiate into endothelial lineage cells, may be recruited from the bone marrow after tissue ischemia, vascular insult, or tumor growth.[7,40,41]

Recruitment, mobilization, and differentiation of CEC and ECP have been positively correlated with increased levels of angiogenic growth factors such as VEGF,[41] angiopoietins,[42] and CXCL12.[43]

The present report shows a significant elevation of VEGF-A, a significant decrease of VEGF-D, and comparable levels of Angio-1 and CXCL12 in advanced pancreatic carcinoma patients in comparison with normal donors. A substantial number of studies have shown elevated circulating VEGF-A levels in cancer patients,[44,45] including pancreatic carcinoma patients.[46,47] Moreover, it has been shown that, in gastric cancer patients, circulating VEGF-D is also below normal levels.[48]

Angio-1, a potent pro-angiogenic factor that induces neovascularization stabilized against vascular leaks, is overexpressed in several tumors, whereas decreased or normal levels have been reported in others.[49]

In agreement with reports by Beerepoot *et al.*[33] in patients with various types of cancer, circulating levels of CXCL12, the principal chemokine that modulates trafficking of hematopoietic stem and progenitor cells including hemangiocytes,[50] was also within the normal range in advanced pancreatic carcinoma patients.

As other studies have found,[10,15,33] in advanced pancreatic carcinoma patients we observed that, among the angiogenic factors we studied, only plasma levels of VEGF-A were correlated with ECP counts, suggesting that VEGF-A is essential for the mobilization of bone marrow-derived ECP. Moreover, whereas VEGF-D, Angio-1, and CXCL12 plasma levels were not correlated with any of the clinical or pathological features studied, interestingly there was a close association between high VEGF-A level, advanced cancer stage, increased risk of poor prognosis, and poor survival. The available data on the prognostic significance of circulating VEGF levels in malignancies is controversial.[46] In gastric cancer, VEGF-A levels have been found to correlate with local tumor extent, disease stage, and the presence of distant metastases; it is also an independent prognostic factor for patient survival.[51]

When specific subpopulations of CEC were correlated with TNM stage, no statistically significant correlation was found, but overall survival was inversely correlated with ECP. Endothelial progenitors are recognized to be key contributors to the first steps of tumor vascularization, suggesting that those patients with higher ECP numbers, presumably having microenvironmental conditions such as inflammation favoring ECP mobilization, develop earlier tumor blood vessels, enabling the tumor to grow and metastasize faster. Indeed, a positive correlation was found between circulating ECP and VEGF-A levels.

Although following different approaches, several studies report a positive correlation between ECP and overall survival in several types of tumor,[33] including small cell lung cancer,[18] breast,[27] and pancreatic carcinoma.[52] However, interlaboratory variability on the definition of ECP and CEC makes comparison among different reports difficult.

Curative surgery is practicable only in a small group of pancreatic carcinoma patients, so systemic palliative chemotherapy remains the standard of care for patients with locally advanced

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or metastatic cancer. In this study, single therapy (GEM) or double-drug combination (GEM + OX, or GEM + 5-FU) were used to treat locally advanced and metastatic pancreatic cancer patients. When the relative effects of different chemotherapy regimens were evaluated at the time of first restaging, GEM alone reduced both VEGF-A levels (in agreement with a previous study)[45] and rCEC counts. The combination of GEM and OX also increased VEGF-D levels to normal values. Many traditional chemotherapy drugs, such as taxanes and thalidomide, are also cytotoxic to CEC.[14] By contrast, in our study, GEM associated with 5-FU [data not shown because of the small number of patients ($n = 3$)] seems not to affect any of the parameters studied. This is not surprising, as we have elsewhere described an antagonistic interaction between these two drugs in some pancreatic cancer cases.[53]

After these cytotoxic agents were given, we did not detect marked mobilization of bone marrow-derived ECP, as has been reported in the case of treatment with certain cytotoxic agents.[10,54] This discrepancy may be due to the fact that our analysis was done after an interval of 2–3 months, depending on the chemotherapy regime, whereas mobilization of ECP appears to be an early event and is most likely connected to the modulation of circulating CXCL12 levels, which we did not observe in our patients after pharmacological treatment. Moreover, in an animal model, only certain drugs, most notably paclitaxel but also 5-FU and docetaxel, were found to cause acute elevations in viable ECP levels within 24 h of a single bolus injection, whereas others, such as gemcitabine, cisplatinum, and doxorubicin, failed to do so.[55]

Gemcitabine-based protocols affected circulating VEGF-A levels and numbers of rCEC, which decreased after treatment, but left intact the ECP compartment. When mature CEC are targeted, blood vessels are presumably destroyed, and thus delivery of therapeutics is compromised. Moreover, this regression of the tumor vasculature, in association with downregulation of VEGF-A (the major factor maintaining the vascular network) drives rebound revascularization and tumor regrowth/recovery after therapy; ECP are key contributors to this regression. The failure of this therapeutic protocol in terms of its inability to control tumor growth may be related to this mechanism.

From our study it emerged that, in pancreatic carcinoma, VEGF-A and ECP may represent reliable prognostic markers, being inversely associated with overall survival. In locally advanced or metastatic pancreatic carcinoma, some standard chemotherapy regimens (GEM or GEMOX) can reduce circulating levels of both VEGF-A and rCEC, but fail to attack ECP. The poor response rate of pancreatic cancer patients suggests that ECP, by providing both instructive (release of pro-angiogenic cytokines) and structural (vessel incorporation and stabilization) functions, may play an important role in facilitating neoangiogenesis and metastasis after chemotherapy intervention. Indeed, when basal VEGF-A, VEGF-D, CXCL12, and Angio-1 plasma levels and rCEC, aCEC, and ECP numbers were considered in terms of clinical response to standard chemotherapy regimens, no significant differences were found between responders (patients entering into partial remission or with stable disease) and non-responders (patients with progressive disease). A potential limitation of these findings could be the different types of chemotherapy considered and the limited number of patients evaluated. However, recent trials evaluating GEM without or with potentially synergistic agents, including OX and 5-FU, showed no statistically significant differences in term of overall survival.[56,57]

Proangiogenic factors are secreted by the tumor cells themselves or by cells in the tumor microenvironment, such as stromal cells and immune cells. During inflammation, activated endothelium produces cytokines and cell adhesion molecules, which recruit inflammatory cells and bring them to the injury site. In turn, inflammatory cells, once within the tumor, are induced to express proangiogenic and tumor-promoting factors.[58]

It has been shown that inflammation, in part, bridges the link between angiogenesis and carcinogenesis in several tumors, including pancreatic carcinoma; chronic pancreatitis has been linked to an increased risk of pancreatic cancer.[59] In our study, chronic pancreatitis was only indicated histologically in one case.

It would thus appear that, rather than looking for the presence of circulating tumor cells, which is a difficult undertaking due to the small numbers of such cells and the lack of distinctive markers, it may be appropriate to look for cell markers indicative of host cells, cytokines, chemokines, and their receptors, as these are easily measured and may represent reliable indicators to predict metastasis and recurrence.[60]

Our clinical study indicates that numbers of CEC and their subpopulations (rCEC, aCEC, and ECP) are elevated in patients with advanced pancreatic carcinoma compared to healthy controls. Endothelial progenitor cell counts, together with circulating VEGF-A plasma levels, are positively correlated to prognosis and disease stage. Thus, ECP may serve as a potential tumor biomarker in pancreatic carcinoma patients to assess tumor burden and predict response to therapy. Importantly, these results point to a new perspective concerning the impact of conventional chemotherapy on tumor angiogenesis, and hence on how combinations with anti-angiogenic drugs may amplify the antitumor effects of chemotherapy. However, the significance of ECP as a potential prognostic and predictive indicator will need to be evaluated prospectively in a large cohort of patients with operable cancer. Endothelial progenitor cell-based therapy may be said to possess great potential in terms of reducing mortality in pancreatic carcinoma patients.

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Abbreviations

5-FU	5-fluorouracil
aCEC	activated circulating endothelial cells
Angio-1	angiopoietin-1
CEC	circulating endothelial cells
CI	confidence interval
CXCL12	chemokine (C-X-C motif) ligand 12
ECP	endothelial progenitor cells
GEM	gemcitabine
OX	oxaliplatin
rCEC	resting circulating endothelial cells
VEGF	vascular endothelial growth factor

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