

Levels of Elevated Circulating Endothelial Cell Decline after Tumor Resection in Patients with Pancreatic Ductal Adenocarcinoma

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Abstract. Aim: To evaluate circulating endothelial lineage cells (ELCs) as biomarkers of tumor neovascularization in patients with pancreatic ductal adenocarcinoma (PDAC). Materials and Methods: ELCs were isolated from the peripheral blood of patients with PDAC (n=14) or controls (n=17) before and after tumor resection/surgery and quantified using flow cytometry. Vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) were detected in tumor using immunohistochemistry and in plasma using an ELISA technique. Results: Circulating ELC levels were increased in patients with PDAC compared to controls. After PDAC resection, ELC levels declined. ELC level increases were associated with cancer recurrence. VEGF and PIGF were identified in cancer cells and exocrine pancreas cells. Only PIGF was detected in tumor-associated inflammatory cells. Plasma levels of PIGF were higher in patients with PDAC compared to controls. Conclusion: Circulating ELCs are a potential biomarker of PDAC neovascularization, and PIGF may be an important target in treatment of PDAC.

Tumor neovascularization is required for cancer growth (1), and anti-angiogenesis agents have been successful in treating advanced cancer (2, 3). As therapeutic agents targeting tumor neovascularization are developed and applied, it is important

to identify biomarkers that can monitor progression/regression of tumor neovasculture. Growth factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) promote new blood vessel formation (4), and elevated levels are associated with cancer (4, 6). Recent data indicate that endothelial lineage cells (ELCs)-including endothelial progenitor cells (EPCs) recruited from bone marrow that incorporate into tumor vasculature (7, 8) and mature circulating endothelial cells (CECs) shed from tumor neovasculture (9) are elevated in cancer patients (10).

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with 5-year survival rates of <5% (11). Antiangiogenic therapy has not demonstrated benefit in patients with PDAC (12). It is uncertain whether this is because other targets play a more important role in this form of cancer or if we are unable to establish an appropriate drug dosing and monitor treatment efficacy in patients with PDAC because of the lack of a reliable biomarker for neovascularization. Circulating ELCs have not been studied in PDAC patients. This study was designed to evaluate circulating ELCs as an indicator of tumor neovascularization in patients with PDAC, hypothesizing that if the tumor is the source of pro-angiogenic growth factors, levels of circulating ELCs should be elevated while tumor is present and decline following tumor resection.

Materials and Methods

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Patient samples. Patients at the New York University Langone Medical Center from November 2006, to October 2007, were recruited for this Institutional Review Board approved study. Clinical information was stored in a HIPAA-compliant, secure computer database. Enrolled patients had no history of chemotherapy, radiation, blood dyscrasia, thrombosis, granulocyte colony stimulating factor (G-CSF) therapy or myocardial

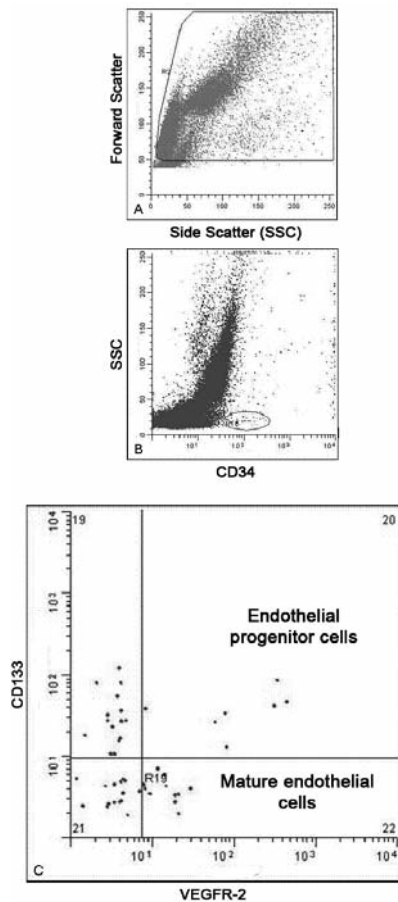


Figure 1. Gating. A: Forward scatter vs. side scatter. B: Elliptical gate identifying live cells from A with low side scatter and CD34⁺. C: Cells from elliptical gate in B. Endothelial progenitor cells (EPCs) are VEGFR2⁺CD133⁺. Mature endothelial cells (CECs) are VEGFR2⁺CD133⁻. Endothelial lineage cells are EPCs⁺CECs.

infarction within 6 weeks of pre-operative evaluation. Fourteen patients with PDAC (7 women, 7 men, aged 48-85 years), 4 with benign lesions (2 women, 2 men, aged 38-80 years), and 13 men scheduled for hernia repair (aged 54-67 years) as controls were recruited.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich St. Louis, MO, USA) (13, 14) from venous blood collected in K₂-EDTA containing Vacutainer tubes (Becton, Dickinson and Company Franklin Lakes, NJ USA). Following red cell lysis with ammonium chloride (Stem Cell Technologies Seattle, WA USA), PBMCs (1.5×10⁶) were Fc-receptor-blocked (Miltenyi Biotec Auburn, CA, USA) and stained in duplicate with fluorescent anti-CD34 (Becton, Dickinson and Company), CD133 (Miltenyi Biotec Auburn, CA, USA), VEGFR2 (R&D Systems Minneapolis, MN USA), and CD14 (eBioscience Inc San Diego, CA, USA) monoclonal antibodies or isotype controls (eBioscience Inc). Cells were incubated 30-45 minutes at 40°C in the dark,

washed twice with Dulbecco's phosphate-buffered saline with 2% fetal bovine serum (Stem Cell Technologies), and fixed in 0.4% para-formaldehyde (15). Cells were evaluated on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) 4-color flow cytometer at the NYU Cancer Institute Flow Cytometry and Cell Sorting Facility.

Data were analyzed using WinList software (Verity Software House Topsham, ME USA). The identity of ELCs is still debated. Data suggest that endothelial EPCs are CD34⁺ VEGFR2⁺ (13, 16). While these markers can also be found on mature endothelial cells, further investigation demonstrates that expression of CD133 identifies EPC amongst cells that are CD34⁺ VEGFR2⁺ (17). In this study, of 200,000 cellular events counted per sample, cells with low side scatter (SSC) were gated; EPCs were identified as CD34⁺ VEGFR2⁺ CD133⁺ and CECs as CD34⁺ VEGFR2⁺ CD133⁻ (13) (Figure 1). The ELC level was defined as the sum of EPCs and CECs divided by total live cells ×100.

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded 5-micron sections were deparaffinized. Antigen was retrieved with Ventana's protease-1 reagent ×2 minutes for VEGF and with a 1200-W microwave oven in 10mM citrate buffer pH 6.0×20 minutes for PIGF. Endogenous peroxidase was blocked with hydrogen peroxide. Antibody incubations and detection were performed on a NEXes instrument (Ventana Medical Systems Tucson, AZ USA) using Ventana's reagent buffer and detection kits.

Overnight incubation with primary rabbit anti-human VEGF (1:250) and goat anti-human PIGF (1:50) (Santa Cruz Biotechnology Santa Cruz, CA, USA) was followed by biotinylated goat anti-rabbit (Ventana Medical Systems Tucson) and 1:50 horse anti-goat antibody (Vector Laboratories, Burlingame, CA, USA), respectively. Streptavidin-horseradish-peroxidase conjugate was applied. Complexes were visualized with 3,3 diaminobenzidine and copper sulfate. Slides were counterstained with hematoxylin, dehydrated, and mounted. Appropriate controls were included.

Slides were analyzed by two pathologists (CH, DZ) blinded to the specimen source. Staining was measured as the overall percentage of cells staining within one high-power field (0=no staining present, 1+≤1%, 2+=1-10%, 3+=11-50%, 4+≥50%). Upon disagreement, consensus was reached while viewing slides at a multiheaded microscope. A Nikon Eclipse E400 microscope (Nikon Melville, NY, USA) with a Spot Insight QE camera (Diagnostic Instruments, Inc, Sterling Heights, MI, USA) was used to take images.

Enzyme-linked immunosorbent assay (ELISA). Plasma was isolated, distributed to microcentrifuge tubes, and stored (-80°C). Human Quantikine Kits (R&D Systems Minneapolis) targeting VEGF or PIGF were used as directed by the manufacturer.

Statistics. Baseline ELC, VEGF, and PIGF levels were compared between PDAC patients and controls. Boxplots provided graphical distribution summaries. Since sample sizes were small and value ranges wide, nonparametric statistical methods were used.

The Wilcoxon signed-rank test was used to compare duplicate ELC measurements and ascertain whether they could be combined for better precision. Since duplicate measurements did not differ, the means of duplicate measurements were used.

ELC level changes from baseline to first follow-up visit (within 7 weeks) were compared between the eight PDAC patients and

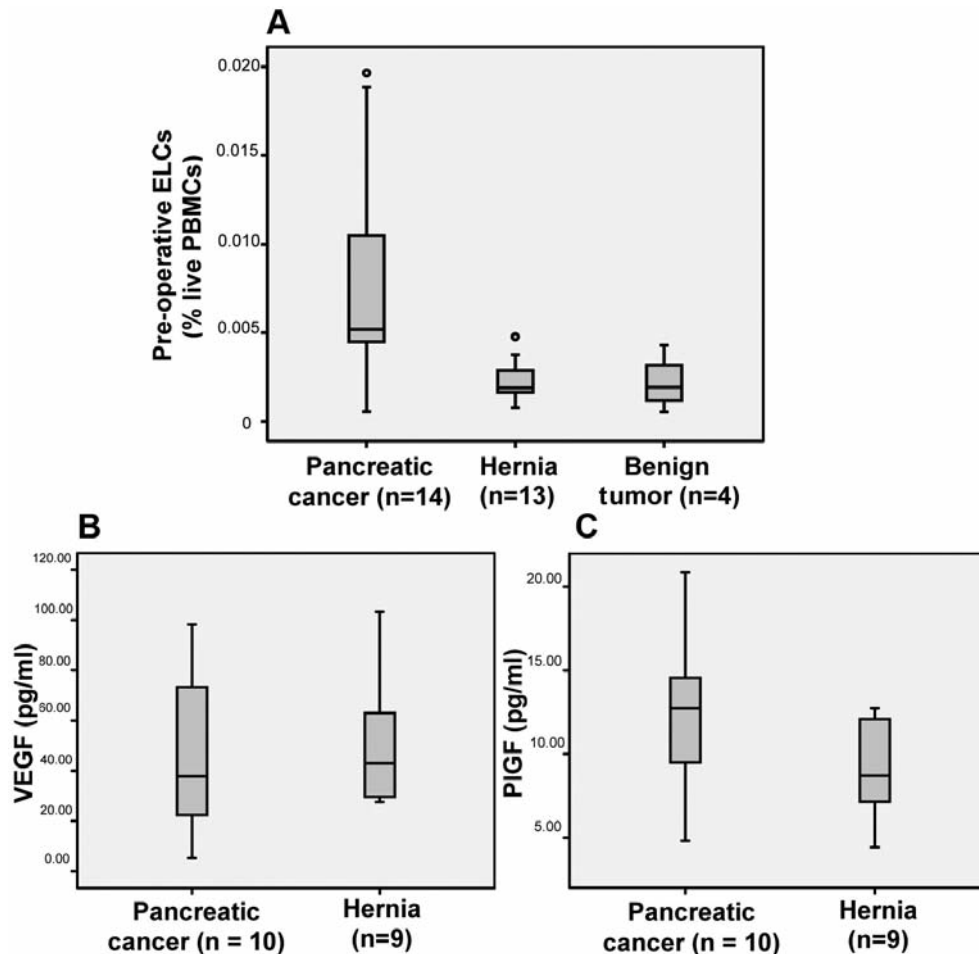


Figure 2. Each boxplot marks the lower and upper quartile of a distribution. The point within the box represents the median. Vertical box-length is one interquartile range (IQR). The middle 50% of the values reside within the box. Vertical lines outside the box terminate at the largest and smallest values beyond the box within 1.5 IQRs from the box. Any point beyond 1.5 IQRs from the box is an outlier. Circles indicate moderate outliers (values located above the third quartile+1.5*IQR) A: Circulating endothelial lineage cell levels were elevated in pancreatic cancer patients compared to surgical controls ($p<0.001$) and to patients with benign pancreatic lesions ($p=0.012$) before surgery. B: There was no difference in pre-treatment plasma levels of VEGF in patients with pancreatic cancer 37.85 pg/ml (IQR=60.11) vs. surgical controls 42.93 pg/ml; IQR=47.23. C: Plasma PlGF was elevated to 12.74 pg/ml (IQR=6.22) for the pancreatic cancer group vs. 8.71 pg/ml (IQR=5.79) for 9 surgical controls ($p=0.053$).

controls who could be followed post-operatively. Percentage change in ELC from baseline (the difference between first follow-up ELC value and baseline ELC value divided by baseline value) was used for comparisons of change in ELC levels from baseline to the first follow-up visit because baseline ELC values differed among the groups.

When three groups were compared, the Kruskal-Wallis test was used at an alpha level of 0.05, if a difference was seen, three Mann-Whitney tests were used for pairwise comparisons between groups run at an alpha level of 0.0167 using the Bonferroni adjustment for multiple comparisons. When two groups were compared, the Mann-Whitney test was used at a significance level of 0.05.

Two-sided tests were performed using SPSS Version 14 (SPSS, Chicago, IL, USA).

Results

Circulating ELC levels are elevated in patients with PDAC before treatment. The median pre-treatment ELC level was 0.0052% [interquartile range (IQR)=0.0076%] for the 14 patients with PDAC, 0.0019% (IQR=0.0014%) for the 13 surgical controls, and 0.0019% (IQR=0.0029%) for the 4 patients with benign pancreatic masses. Pre-treatment ELC levels differed among the three groups ($p=0.001$), specifically PDAC patients vs. surgical controls ($p<0.001$) and PDAC patients vs. patients with benign masses ($p=0.012$). There was no difference between the surgical controls and patients with benign pancreatic masses (Figure 2).

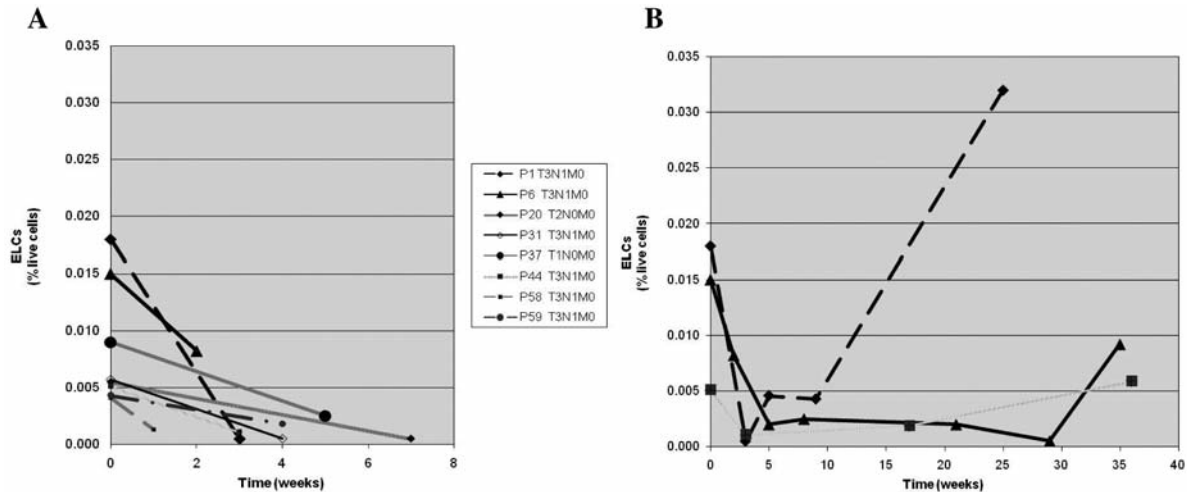


Figure 3. Circulating endothelial lineage cells (ELCs) in patients with pancreatic ductal adenocarcinoma. A: ELCs in patients with pancreatic cancer before and after tumor resection ELC levels in 8 patients with pancreatic cancer declined after resection. This decline was more pronounced compared to that of surgical controls ($p=0.006$). B: ELC levels in 3 patients with pancreatic cancer increased. Within 9 months of follow-up, these levels were associated with cancer recurrence.

Circulating ELC levels decline in patients with PDAC after tumor resection. Of 14 patients with PDAC, 8 had resectable lesions. In 8/8, circulating ELC levels declined in the interval between surgery and the first postoperative visit (within 7 weeks after tumor resection: $p=0.012$) before adjuvant treatment was initiated, and the values reflect only surgical tumor reduction. The circulating ELC level declines ranged from 0.0030% to 0.018% (median=0.0047%) (Figure 3A). Percentage changes in ELC levels after cancer resection differed from that of the 7 surgical controls and 4 patients with benign pancreatic masses who were followed post-operatively (0.006). Specifically, there were differences in percentage change of ELC between patients with resected PDAC and surgical controls ($p=0.009$), as well as between patients with resected PDAC and resected benign pancreatic masses ($p=0.004$). There was no difference in the percentage change between patients with resected benign pancreatic masses and surgical controls.

Circulating ELCs in patients with PDAC during follow-up over time: ELC levels increase with disease progression. Seven out of eight patients with resected PDAC were followed for ≥ 6 months post-resection (Figure 3B). P37 was not available for follow-up after 11 weeks post-resection secondary to an acute myocardial infarction five months post-surgery. Within 9 months, 3/8 patients eventually demonstrated elevations in their circulating ELC levels more than 4 \times their lowest post operative measurements (P1, P6, P44). These increases were associated with cancer recurrence confirmed by radiological study (Figure 3B), either computed tomography (CT) or positron-emitted tomography (PET)-CT of the chest, abdomen, and pelvis. Of the remaining 5, 4 had

no evidence of disease recurrence. Chemotherapy regimens at the time of blood acquisition were unchanged.

VEGF and PlGF are expressed differentially in the PDAC microenvironment. IHC sections from resected PDAC were analyzed semiquantitatively for VEGF and PlGF to study the expression of these factors in the tumor microenvironment. Histological sections of normal pancreas demonstrated VEGF predominantly in ductular and acinar cells and PlGF in islets of Langerhans. In all resected PDACs, tumor cells stained for VEGF and PlGF (Figure 4). A total of 5/8 tumors had $\geq 3+$ staining for these factors. Exocrine pancreas within tumor-associated chronic pancreatitis stained for VEGF (3-4+) and PlGF (2-4+). Tumor-associated inflammatory cells demonstrated minimal to no staining for VEGF (0-1+), although they stained for PlGF (2-3+) in the same regions.

VEGF and PlGF in plasma. PlGF is elevated in plasma of patients with PDAC. Flow cytometry and IHC data support the hypothesis that tumor is a source of pro-angiogenic growth factors. We used archival platelet-depleted plasma samples obtained from our tissue bank to detect VEGF and PlGF via ELISA in patients with PDAC ($n=10$) and surgical controls ($n=9$) (Figure 2). There were no differences in VEGF plasma levels between the two groups. Median pre-treatment VEGF plasma levels were 37.85 pg/ml (IQR=60.11) for the PDAC group and 42.93 pg/ml (IQR=47.23) for controls. The median pre-treatment PlGF plasma level was elevated to 12.74 pg/ml (IQR=6.22) for the PDAC group compared to 8.71 pg/ml (IQR=5.79) for surgical controls ($p=0.053$). This is consistent with recent data (18).

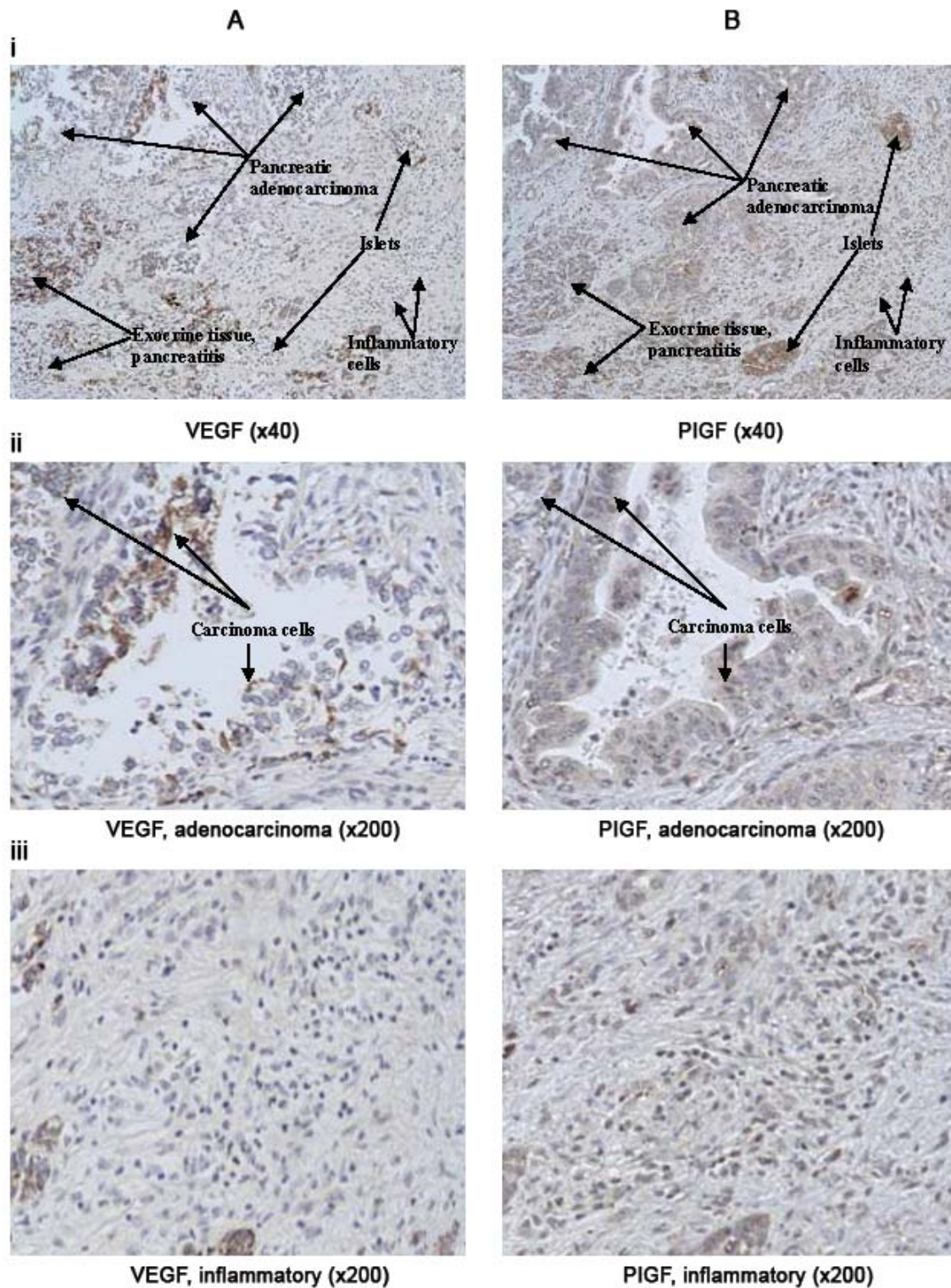


Figure 4. Expression of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) primary antibody in pancreatic ductal adenocarcinoma. A: Anti-VEGF-stained section (i), $\times 40$ magnified view. Cancer cells and exocrine tissue within chronic pancreatitis staining positively. Islets and inflammatory cells, absent to minimal staining. (ii): $\times 200$ Magnified view of carcinoma cells from (i), note positive anti-VEGF staining. (iii) $\times 200$ Magnified view of inflammatory cells from (i), note a lack of anti-VEGF staining. B: Anti-PIGF-stained section, (i) $\times 40$ magnified view. Cancer cells, exocrine tissue within chronic pancreatitis, and islets staining positively. (ii) $\times 200$ Magnified view of carcinoma cells from (i), note positive anti-PIGF staining. (iii) $\times 200$ Magnified view of inflammatory cells from (i), note positive anti-PIGF staining.

Discussion

These data suggest that ELCs are elevated in the peripheral blood of patients with PDAC before surgical resection is performed. As the ELC levels were found to decline post-resection, these data substantiate the hypothesis that growth factors elaborated by PDAC support the recruitment of endothelial lineage cells into the neovasculature. Following tumor resection, the source of growth factors is removed, at least transiently, until another source (recurrent tumor) develops sufficiently to mobilize ELCs. In three patients with PDAC recurrence, ELC levels increased; these post-surgery longitudinal data suggest that in PDAC patients, tumor is the source of pro-angiogenic factors which stimulate ELC presence in the circulation. Therefore, ELCs may serve as a marker for neovascularization in patients with PDAC, and they may be used to monitor post-surgical outcome.

Mobilization of EPCs from the bone marrow can be stimulated by both VEGF and PlGF (6, 19). PlGF acts synergistically with small amounts of VEGF to promote tumor angiogenesis (20, 21). Immunohistochemical data from this study indicate that both VEGF and PlGF are expressed in the tumor microenvironment and that tumor-associated inflammatory cells may contribute to neovascularization in pancreatic cancer through secretion of PlGF. When evaluating VEGF and PlGF in the peripheral blood of patients with PDAC, it was discovered that PlGF, not VEGF, was elevated in patients with PDAC. Realizing that bevacizumab, which targets only VEGF, does not improve survival in patients with pancreatic cancer (12), our findings suggest that targeting both VEGF and PlGF and/or their receptors may potentially enable successful anti-angiogenic therapy in patients with PDAC.

In conclusion from these early observations, ELC levels are elevated in patients with pancreatic ductal adenocarcinoma and are potential biomarkers of neovascularization. The cancer microenvironment supports neovascularization, and an elevation in PlGF may be important to neovascularization of PDAC and a significant additional target for successful anti angiogenesis therapy of this form of lethal cancer.

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