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Expression of Lymphangiogenic Factors and Evidence of Intratumoral Lymphangiogenesis in Pancreatic Endocrine Tumors

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Lymphangiogenesis is thought to promote the progression of malignant tumors. Because the lymphangiogenic factors vascular endothelial factor (VEGF)-C and -D are expressed in endocrine cells, we investigated their expression in pancreatic endocrine tumors (PETs) and correlated these data and intratumoral lymph vessel density (iLVD) with clinicopathological features. Lymph vessels were identified with anti-podoplanin antiserum and with podoplanin/proliferating cell nuclear antigen double labeling. PETs (n = 104) were investigated by immunohistochemical staining for VEGF, basic fibroblast growth factor, and VEGF-C expression. VEGF-C and VEGF-D mRNA were quantified by real-time reverse transcriptase-polymerase chain reaction. PETs showed higher iLVD than normal pancreata, but iLVD did not discriminate between benign and malignant PETs. In PETs proliferating lymph vessels were identified. High iLVD was associated with lymph vessel invasion and it was more frequent in angioinvasive/metastatic tumors than in grossly invasive tumors. VEGF-C expression correlated with iLVD as well as with glucagon and pancreatic polypeptide expression. PETs show intratumoral lymphangiogenesis, which is associated with VEGF-C expression in tumor cells. The association between iLVD and lymph vessel invasion and angioinvasive/metastatic features in PETs suggests that lymphangiogenesis may promote malignant progression of PETs. PET is the first human tumor entity in which VEGF-C-related intratumoral lymphangiogenesis has been demonstrated. *(Am J Pathol 2004, 165:1187–1197)*

The biological behavior of pancreatic endocrine tumors (PET) is difficult to predict on the basis of histological criteria. In the absence of clear signs of malignancy, such as invasion of adjacent organs, angioinvasion, or metastasis, the prognosis remains uncertain. Because most human carcinomas metastasize via lymphatic invasion, lymph node metastasis is a key prognostic factor for the clinical outcome. By which mechanism tumor cells spread through the lymph vessels is unknown. One proposed mechanism is the induction of new lymph vessels by tumor or inflammatory cells, facilitating lymphangioinvasion. Intratumoral lymph vessels have been detected in head and neck squamous cell carcinomas[1,2] and in cutaneous melanomas.[3,4] In squamous cell carcinomas intratumoral lymph vessel density (iLVD) has been shown to correlate with lymph node metastasis, whereas the results for melanomas are inconsistent. Beasley and colleagues[1] and Straume and colleagues[3] showed that intratumoral lymphatic endothelial cells were capable of proliferation, suggesting *de novo* lymphangiogenesis. No evidence of intratumoral lymph vessels was found in ovarian,[5] liver,[6] breast,[7,8] or cervical carcinomas.[9]

Vascular endothelial growth factor (VEGF)-C and VEGF-D are cytokines possessing structural homology with VEGF and platelet-derived growth factor.[10–12] They are secreted glycoproteins, both of which can act through the tyrosine-kinase receptors VEGF-R2 and VEGF-R3.[11,13] Because VEGF-R3 is almost exclusively expressed in lymphatic endothelial cells, VEGF-C and VEGF-D stimulate the growth of these cells and thus induce lymphangiogenesis.[11,13–15] VEGF-C expression has been reported in several types of cancer, such as carcinomas of the esophagus, stomach, colorectum,[16] head and neck,[1,2] breast,[17–19] and cervix[9] and in melanomas.[3] These studies demonstrated that tumor cells or

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Table 1. Clinicopathological Features of 111 PETs

	No. of tumors	Gender		Age (years)		Tumor size*			
		F	M	Median	SD	Range	Median	SD	Range
Functionally active									
Benign	32	19	13	48	19.6	17–88	1.5	0.42	0.5–2
Malignant	25	14	11	51	15.2	24–79	4	2.46	2–11
Functionally inactive									
Benign	7	5	2	61	14.8	35–75	2	1.67	0.7–6†
Malignant	40	23	17	57	14.2	3–75	5	3.03	2–12
Probably malignant‡	7	5	2	50	14.7	17–59	5	3.6	2.2–12
Total	111	66	45	54	16.26	3–88	3	3.04	0.5–12

*Maximum diameter in cm.
†This group contains one large cystic tumor (6 cm) that had already been noted 10 years before the surgical resection and showed no signs of malignant transformation.
‡This group was included only to test the correlation between glucagon expression, VEGF-C expression, and LVD.

tumor-associated macrophages served as a cellular source of VEGF-C. VEGF-D expression was detected in colorectal,[20–22] ovarian,[23] endometrial,[24] and breast carcinomas,[18,25] and in glioblastomas[26] and melanomas.[27]

In nonneoplastic cells, VEGF-C and VEGF-D were found to be expressed mainly in the neuroendocrine cell system, often in close proximity to fenestrated vessels positive for VEGF-R2 and VEGF-R3. VEGF-C was identified in pituitary cells, pancreatic α-cells, adrenal medullary cells, and serotonin-producing cells of the gastrointestinal tract, whereas VEGF-D was detected in the cortex of the adrenal gland and in gastrin-producing cells.[28]

Because malignant PETs frequently exhibit lymphatic invasion and lymph node metastases, we investigated whether these tumors are capable of inducing lymphangiogenesis that may promote tumor progression. On the basis of the distribution pattern of lymphangiogenic factors in normal endocrine cells, we hypothesized that PETs may express VEGF-C or VEGF-D and that this expression is related to lymphangiogenesis and biological features.

Materials and Methods

Tissues

Formalin- or Bouin-fixed paraffin-embedded tissue blocks from 111 PETs from the period 1972 to 2002 were investigated. They were retrieved from the consultation files of the Department of Pathology of the University of Kiel. Only cases with complete patient records, including gender, age, clinical manifestation, size and localization of tumor, and presence of metastasis were considered. Table 1 summarizes the most important clinicopathological characteristics of the investigated PETs. All benign functionally active PETs were insulinomas. Two of them arose in patients with multiple endocrine neoplasia type I. The malignant functionally active PETs consisted of 11 insulinomas, 7 gastrinomas, 4 glucagonomas, 2 VIPomas, and 1 ACTH-producing tumor. Clinical symptoms and elevated serum hormone levels defined functionally active PETs. PETs that were $\leq$2 cm in diameter and showed no signs of invasive growth or metastasis were considered benign. Malignant PETs showed blood or lymph vessel invasion (detected with endothelial cell-specific immunostains), tumor infiltration of adjacent organs, or histologically verified lymph node or blood-borne metastases. To increase the number of glucagon-expressing tumors, seven glucagon-expressing PETs (one glucagonoma, six functionally inactive) that lacked signs of malignancy but were larger than 2 cm were also included in the study. Immunohistochemically, all tumors expressed synaptophysin and/or chromogranin and were well differentiated according to the World Health Organization classification.[29]

Freshly cryoconserved tumor tissue from six pancreatic PETs served as source of reference RNA for the quantitative polymerase chain reaction (PCR) investigations. These tumors were collected according to the protocol approved by the ethics committee of the University of Kiel Hospitals (permission number, 110/99). This protocol prescribed signed informed consent by all patients.

Normal pancreatic tissues removed from 12 individuals who died of suicide were obtained from the Department of Forensic Medicine of Semmelweis University (Budapest, Hungary). The pancreata were removed within 1 hour after death with the permission of the local Ethical Commission for Scientific Research (permission number, 140-1/1996) and immediately fixed in 4% buffered formalin. Histological examination of the pancreas tissues revealed no signs of pancreatic disease. During the forensic autopsy no signs of organic disease affecting the pancreas were seen.

Immunohistochemistry

Tumor tissues were fixed in formalin or in Bouin's fixative and routinely processed for paraffin sectioning. Three-μm-thin paraffin sections were deparaffinized, rehydrated, and immunohistochemical stains were performed according to routine methods. Before application of the primary antibody, blocking with nonimmune serum was performed for 20 minutes. Table 2 lists the positive controls, demasking methods, antibodies, and detection systems. For negative controls, the primary antibodies were omitted or the primary anti-basic fibroblast growth factor antibody was incubated with specific blocking peptide in 10-fold molar excess before the staining. For the VEGF-D

Table 2. Antibodies, Conditions, and Controls for Immunohistochemical Reactions

Antigen	Primary antibody clone/code	Source	Concentration/ dilution	Antigen demasking	Secondary antibody	Detection system	Positive control
Synaptophysin	Rabbit/A0010	Dakocytomation, Glostrup, Denmark	6 μg/ml	Pressure cooker, TEC buffer 3 minutes	Rabbit Vectastain peroxidase kit; Vector Laboratories, Burlingame, CA		Pancreas
Chromogranin	LK2H10/E001	LINARIS, Wertheim-Bettingen, Germany	1:2	–	Mouse Vectastain peroxidase kit, Vector Laboratories		Pancreas
Insulin	HB125/02911	Biogenex, San Ramon, CA	1:40	–	Mouse Vectastain peroxidase kit, Vector Laboratories		Pancreas
Glucagon	Rabbit/039P	Biogenex	1:60	–	Rabbit Vectastain peroxidase kit, Vector Laboratories		Pancreas
Pancreatic polypeptide	Rabbit	R.E. Chance, Indianapolis, IN	1:5000	–	Rabbit Vectastain peroxidase kit, Vector Laboratories		Pancreas
Somatostatin	Rabbit/A0566	DAKO	1:200	–	Rabbit Vectastain peroxidase kit, Vector Laboratories		Pancreas
Gastrin	Rabbit	Paesel, Frankfurt, Germany	1:3000	–	Rabbit Vectastain peroxidase kit, Vector Laboratories		Duodenum
Vasoactive intestinal peptide	Rabbit/18-0080	ZYMED, San Francisco, CA	1:10	–	Rabbit Vectastain peroxidase kit, Vector Laboratories		Functionally active VIPoma
CD34	QBEND10/0786	Immunotech, Marseille, France	1 μg/ml	–	Mouse Vectastain peroxidase kit, Vector Laboratories		Endothelial cells
Podoplanin	Rabbit	[32]	1:2000	Pressure cooker, TEC buffer, 3 minutes	Rabbit Envision kit, DAKO		Lymphangiom
VEGF	Goat/AF293 NA	R&D Systems, Wiesbaden, Germany	10 μg/ml	Pressure cooker, TEC buffer, 3 minutes	Biotin conjugated anti-goat IgG, 705-065-147; Jackson Laboratories, West Grove, PA	ABC Elite Kit, Vector Laboratories	Fetal kidney
VEGF-C	Goat/AF752	R&D Systems	0.5 μg/ml	Pressure cooker, Glycol containing solution,* 5 minutes	Biotin conjugated anti-goat IgG, 705-065-147; Jackson Laboratories	ABC Elite Kit, Vector Laboratories	α-Cells in Langerhans-islets
VEGF-D	Mouse/MAB 286	R&D Systems	15 μg/ml, overnight	Pressure cooker, Glycol containing solution,* 5 minutes	Mouse Envision kit, DakoCytomation		Gastrin-producing cells of stomach
bFGF	Rabbit/sc-79	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1 μg/ml	Pressure cooker, TEC buffer, 3 minutes	Rabbit Vectastain peroxidase kit, Vector Laboratories		Chronic pancreatitis

*Biologo, Kiel, Germany.

staining, the primary antibody was replaced with control mouse IgG2a (DAKO Cytomation, Glostrup, Denmark).

Expression of hormones, VEGF, VEGF-C, VEGF-D, and bFGF was semiquantitatively evaluated as mild (<10% of tumor cells stained), moderate (10 to 50%), or strong (>50%) by one investigator (B.S.) without knowledge of the other tumor features. Hormone expression was carefully analyzed in consecutive sections to avoid misinterpretation of entrapped pancreatic islets. iLVD and peritumoral lymph vessel density (pLVD) were determined by the hot spot method.[30] All areas (0.75 mm^2) with high lymph vessel density stained with anti-podoplanin antiserum were photographed with a Nikon Coolpix 990 digital camera mounted on an Axioskop 50 microscope (Zeiss, Oberkochen, Germany). Intratumoral hot spots in all PETs and peritumoral hot spots (areas in the fibrous capsule of the tumor or in peritumoral tissues without close relationship to tumor cells) in 80 PETs were evaluated. The localization of the 349 digitalized intratumoral hot spots was recorded as peripheral (within 4 mm of the tumor border) or central. Podoplanin-positive vessels with or without lumen were counted with Scion Image Software (Scion Corp., Frederick, MD). Vessels were considered to be invaded if vascular spaces lined by podoplanin-positive (lymph vessel) or CD34-positive (blood vessel) endothelial cells contained definite tumor cell complexes. LVD was determined with the same method in 12 normal pancreata (one section from the head, body, and tail from each pancreas) and peripancreatic fat tissues (one section from each pancreas). Overall 244 hot spots were analyzed in normal pancreatic and peripancreatic tissue.

Table 3. Oligonucleotide Primers and Probes Used for Quantitative PCR

Target	5′ Primer	3′ Primer	Probe	Size of amplicon (bp)	Annealing temperature
VEGF-C[1]	5′-TCAAGGACAGAAGAGACTATAAAATTTGC	5′-ACTCCAAACTCCTTCCCCACAT	6FAM-ATACACACCTCCCGTGGCATGCATTGT-TAMRA	137	60
VEGF-D	5′-TACTCTTCCCCAGCTCACTG	5′-TACTCTTCCCCAGCTCACTG	6FAM-CAAAGAACTCAGTGCAGCCCTAGAGAAACGTTAMRA	116	60
GAPDH[31]	5′-ATTCCACCCATGGCAAATTC	5′-TGGGATTTCCATTGATGACAAG	VIC-CAAGCTTCCCGTTCTCAGCC-TAMRA	72	60

Double Immunolabeling

Tissue sections were incubated in 1% sodium borohydride in phosphate-buffered saline for 30 minutes to reduce autofluorescence and subsequently stained with 1% Sudan Black B (Sigma, Taufkirchen, Germany) for 10 minutes. Nonspecific binding sites were blocked in blocking buffer containing 0.1% bovine serum albumin and 0.2% glycine in Tris-buffered saline for 1 hour at room temperature. Incubation of antibodies was performed overnight in Tris-buffered saline. Primary antibody dilutions were as follows: rabbit anti-podoplanin antiserum, 1:400; monoclonal anti-proliferating-cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA), 1:300; mouse monoclonal anti-glucagon antibody, 1:30; and goat anti-VEGF-C antibody, 1:50. After extensive washing in Tris-buffered saline, secondary antibodies were incubated for 1 hour at 37°C (goat anti-rabbit Alexa Fluor 488, 1:1000; goat anti-mouse Alexa Fluor 546, 1:1000; donkey anti-goat Alexa Fluor 488, 1:1000; all from Molecular Probes, Eugene, OR). Sections were counterstained for nuclei using Hoechst A33528 dye at a concentration of 0.025 mg/ml. After extensive washing in Tris-buffered saline, sections were mounted using ProLong anti-fade mounting medium (Molecular Probes). Laser-scanning analysis was performed using a Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss Jena, Jena, Germany). All recordings were done using multitracking with ×400 original magnification and pinhole diameter set at 1.0 Airy unit.

For colorometric double labeling, PCNA immunostaining was developed with alkaline phosphatase-conjugated secondary antibody and podoplanin staining was developed with peroxidase-conjugated secondary antibody using an Envision double-labeling kit (DAKO Cytomation) according to the manufacturer's instructions. The rate of the double-labeled vessels was determined by counting the nuclei of intratumoral podoplanin-positive microvessels (100 to 350 nuclei in each tumor).

RNA Extraction and Quantitative PCR

Paraffin blocks from 29 formalin-fixed PETs, which consisted of >90% tumor tissue were selected for quantitative PCR. Immunohistochemically, 10 tumors showed no or low VEGF-C expression; 19 tumors revealed moderate or strong VEGF-C positivity. Ten 5-μm-thin sections were cut into tubes, deparaffinized, washed in ethanol, air-dried, and resuspended in 80 μl of 60 mg/ml Proteinase K (Sigma) plus 720 μl of digestion buffer as described by Godfrey and colleagues[31] overnight at 55°C in a shaker. RNA extraction was performed with an RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To remove remnants of genomic DNA, extracted RNA was treated with RNase-free DNase Set (Qiagen) for 20 minutes. The RNA concentration was determined by spectrophotometer (Spectramax 250; Molecular Devices, Sunnyvale, CA) at 260 nm. The purity of the RNA extracts was monitored by OD 260/280 measurement. RNA extracts were aliquoted and stored at −20°C. The cryoconserved tissues were cut into pieces, transferred to tubes, homogenized by pellet pestles, pushed through a 21-gauge needle, and subjected to RNA extraction with the same method.

Primers were designed to span introns in the genomic DNA. Primers and probes for quantitative PCR are listed in Table 3. The levels of VEGF-C and VEGF-D mRNA were quantified by real-time reverse transcriptase (RT)-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystem, Foster City, CA). One-step RT-PCR (QuantiTect Probe RT-PCR, Qiagen) was performed using 100 ng of RNA, 10 pmol of forward and reverse primer, respectively, and 3 pmol of probe in each reaction. The mRNA for VEGF-C and VEGF-D and the control GAPDH were each amplified in separate tubes. VEGF-C and VEGF-D quantification was performed in triplicate and in duplicate, respectively. DNA for the PCR standard was obtained from RNA from fresh PET tissue by RT-PCR using the same primers as for the quantitative RT-PCR. Standard curves were constructed with 10-fold serial dilutions of gel-purified DNA. After performing a linear regression analysis for the standard dilutions the values for the experimental samples were extrapolated and expressed as corresponding attomol of the standard DNA. The values in attomol for VEGF-C and VEGF-D were divided by the value for GAPDH.

Statistics

The association between LVDs (continuous variable) and clinicopathological features or cytokine expression was analyzed with the Mann-Whitney *U*-test and the Kruskal-Wallis *H*-test. Correlations between VEGF-C expression and other tumor parameters were determined with Fisher's exact test. iLVD and mRNA expression levels determined by quantitative PCR were divided into quartiles before their association with VEGF-C protein expression was tested using a one-sided Kendall-Tau b test. Comparisons between continuous variables were made with

Spearman's rank test. Linear regression analysis was applied for testing LVD influencing factors. Logistic regression analysis was performed to test cause variables for VEGF-C expression. P values <0.05 were considered to be significant. All statistical tests were performed using SPSS 10.1 software (SPSS, Chicago, IL).

Results

Lymph Vessel Density in PETs and Normal Pancreatic and Peripancreatic Tissues

Podoplanin marked thin-walled vessels[32] without recognizable muscle layer or pericytes corresponded morphologically to lymph vessels whereas other cell types did not show podoplanin expression. The vast majority of the lymph vessels in normal pancreata had wide open lumina and were in close proximity to ducts, blood vessels, or nerves (Figure 1a). The majority of the intratumoral lymph vessels were collapsed. Lymph vessels were unevenly distributed throughout the tumor. Eighty-five percent of the lymph vessel hot spots were located in a rim of 4 mm at the periphery of the tumors (Figure 1b).

Median LVDs for normal pancreas were 11.5/0.75 mm^2 (mean, 11.9; SD, 3.3; range, 6 to 19), for peripancreatic adipose tissue 8.5/0.75 mm^2 (mean, 9.4; SD, 3; range, 6 to 14), iLVD 16/0.75 mm^2 (mean, 20.4; SD, 16.9; range 0 to 87), and pLVD 14/0.75 mm^2 (mean, 15.5; SD, 7.6; range 4 to 45) for all PETs. Comparison of LVDs in normal tissues versus tumors revealed a significant increase of both iLVD (Mann-Whitney U-test, $P = 0.004$) and pLVD (Mann-Whitney U-test, $P = 0.001$) in PETs. Tumor size was not related to iLVD (Kendall-Tau b test; correlation coefficient, -0.31; $P = 0.675$), but showed a positive correlation with pLVD when tumor size was divided into quartiles and tested with Kendall-Tau b test (correlation coefficient, 0.295; $P = 0.001$). There was no correlation between iLVD and pLVD (Spearman's rank test, $P = 0.582$). Eleven PETs did not show intratumoral podoplanin-expressing lymph vessels. In these cases lymphatics in the surrounding tissue served as positive control to rule out failure of the immunohistochemical detection method.

Table 4 summarizes the correlations between LVDs and clinicopathological features and hormone and cytokine expression. iLVD showed no correlation with biological behavior or functional activity of PETs (Kruskal-Wallis analysis, $P = 0.96$). High to moderate glucagon expression correlated with iLVD (Mann-Whitney U-test, $P < 0.001$), but no other hormone expression in the tumor cells did. If the glucagon-expressing tumors were divided into expressing and nonexpressing subgroups, the significance of the glucagon-iLVD association remained high (Mann-Whitney U-test, $P < 0.001$). Similar results were obtained if the seven additional, probably malignant, glucagon-expressing PETs were included in the analysis. There was a clear association between iLVD and the presence of lymph vessel invasion (Mann-Whitney U-test, $P = 0.004$) (Figure 1c), but not of lymph node metastasis (Mann-Whitney U-test, $P = 0.39$). Interestingly, iLVD exhibited a significant correlation with VEGF-C expression (Mann-Whitney U-test, $P < 0.001$), without being associated with VEGF or bFGF expression. In a multivariate linear regression analysis, moderate to strong glucagon expression ($r^2 = 0.21$; $P < 0.001$; B value, 28.7; 95% confidence intervals, 17.7 to 39.7) and VEGF-C expression ($r^2 = 0.064$; $P = 0.003$; B value, 11.5; 95% confidence intervals, 3.9 to 19.2) remained independent factors of high iLVD.

When the malignant PETs were divided into groups consisting of tumors that infiltrated surrounding organs without showing other signs of malignancy ($n = 7$; largest diameter, 7.5 ± 3 cm) and tumors that were angioinvasive/metastatic ($n = 58$; largest diameter, 5.17 ± 2.77 cm), no tumors in the group consisting of grossly invasive tumors revealed more than 20 iLVD, whereas in the second group 26 of 58 (44.8%) tumors had iLVD values >20 ($P = 0.022$, Fisher's exact test).

Peritumoral LVD values were significantly higher in malignant than in benign PETs (Mann-Whitney U-test, $P = 0.004$), in functionally inactive versus active PETs (Kruskal-Wallis test, $P = 0.001$), in PETs showing high VEGF-C expression versus PETs with no or low VEGF-C expression (Mann-Whitney U-test, $P = <.001$), and in PETs with no or low insulin expression versus moderate-to-strong insulin expression (Mann-Whitney U-test, $P = <.001$). Multivariate linear regression analysis revealed that functional inactivity ($r^2 = 0.19$; $P < 0.001$; B value, 6.6; 95% confidence intervals, 3.5 to 9.6), moderate-to-strong VEGF-C expression ($r^2 = 0.065$; $P = 0.011$; B value, 4.7; 95% confidence intervals, 1.1 to 8.3), and large tumor size ($r^2 = 0.039$; $P = 0.041$; B value, 1.6; 95% confidence intervals, 0.07 to 3.2) increased pLVD independently. pLVD was not related to lymph vessel invasion, lymph node metastasis, and angioinvasive/metastatic phenotype (Mann-Whitney U-test, $P = 0.312$). To detect and quantify proliferating lymph vessels, 27 PETs having high iLVD (>25/0.75 mm^2) were investigated with podoplanin and PCNA immunohistochemistry (Figure 1d). Double-labeled lymph vessels were found in 11 of 27 tumors. The proliferation rate ranged from 0.7 to 3%. We confirmed the co-expression of PCNA and podoplanin by immunofluorescent labeling in eight of nine PETs showing high iLVD (Figure 1e).

VEGF-C, VEGF-D, VEGF, and bFGF Expression

Immunohistochemically, mainly tumor cells and some peritumoral pancreatic islets exhibited VEGF-C expression (Figure 1, f and g). Twenty-one of one hundred four PETs (20.1%) expressed VEGF-C moderately or strongly. Table 5 summarizes the associations between VEGF-C and tumor features. VEGF-C expression was positively correlated with malignant phenotype (Fisher's exact test, $P = 0.002$), moderate to strong glucagon expression (Fisher's exact test, $P = 0.001$), and moderate to strong pancreatic polypeptide expression (Fisher's exact test, $P = 0.016$) in tumor cells and inversely correlated with insulin expression (Fisher's exact test, $P = 0.001$). The correlation between VEGF-C and glucagon expression remained strong (Fisher's exact test, $P < 0.001$) when the tumors were divided into glucagon-negative and -posi-

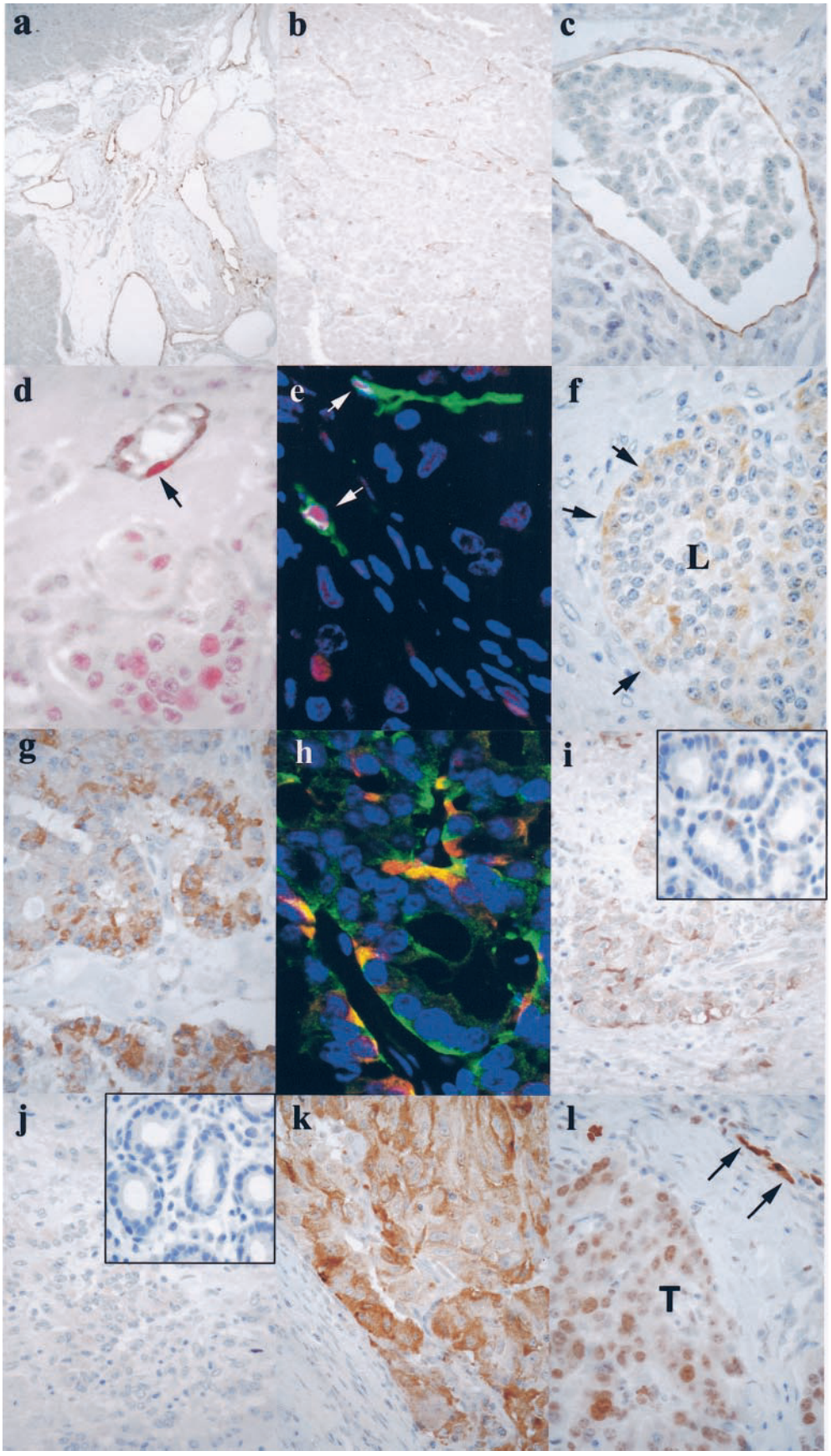
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Table 4. Lymph Vessel Densities Related to Clinicopathological Features and Cytokine Expression in 104 PETs

	Intratumoral lymphatic vessel density				Peritumoral lymphatic vessel density			
	No. of tumors	LVD (mean)	SD	*P* value*	No. of tumors	LVD (mean)	SD	*P* value*
Biological behavior								
Benign all	39	19.5	14			30	12.8	5.9
Malignant all	65	20.9	18.4	0.92	51	17.2	8	0.004
Functionally active								
Benign	32	20	14.8		25	11.2	3.5	
Malignant	25	20.3	14.8		19	14.4	4.8	
Functionally inactive								
Benign	7	17.1	10.6		5	20.8	9	
Malignant	40	21.2	20.6	0.96†	32	18.9	9.1	0.001†
Lymph vessel invasion§								
Absent	45	16.6	16		37	17.6	8.3	
Present	20	30.4	20.3	0.004	14	16.1	7.1	0.69
Lymph node metastasis§								
Absent	41	22.9	20.4		34	15.7	6.7	
Present	24	17.4	14.1	0.39	17	20.2	9.7	0.14
Hormone expression in tumor cells								
Insulin								
Absent/weak	69	21.1	18.3		54	17.6	7.9	
Moderate/strong	35	18.9	13.6	0.93	27	11.3	4.3	<0.001
Glucagon								
Absent/weak	96	18.2	14.4		74	15.6	7.9	
Moderate/strong	8	46.9	22.1	<0.001	7	15.3	2.9	0.65
Absent	64	15.9	13.6		48	14.7	8.6	
Present	40	27.6	19.1	0.001	33	16.8	5.6	0.108
Glucagon‡								
Absent/weak	99	18.9	14.9		77	15.4	7.8	
Moderate/strong	12	37.4	24.3	0.006	11	21.3	9.3	0.03
Absent	64	15.9	13.6		48	14.7	8.6	
Present	47	27.7	18.9	0.001	40	17.9	7.3	0.06
Somatostatin								
Absent/weak	96	21.2	17.2		75	15.2	7.5	
Moderate/strong	8	10.1	5.9	0.076	6	19.8	7.5	0.12
Pancreatic polypeptide								
Absent/weak	94	20.6	17.2		71	15.1	7.5	
Moderate/strong	9	18.2	14.7	0.83	9	18.2	7.5	0.23
Gastrin								
Absent/weak	100	20.5	17.1		79	15.6	7.6	
Moderate/strong	4	16	11.5	0.83	2	13	8.4	0.73
VEGF-A expression								
Absent/weak	26	21.3	14.6		18	13.1	6.2	
Moderate/strong	78	20.3	17.5	0.39	63	16.3	7.9	0.09
VEGF-C expression								
Absent/weak	83	17.2	14.5		62	14.1	7.1	
Moderate/strong	21	34.1	18.7	<0.001	19	20.4	7	<0.001
bFGF expression in tumor cells								
Absent/weak	66	18.2	14.1		49	15.2	8	
Moderate/strong	38	24.6	20.2	0.18	31	16.2	7.1	0.38
bFGF expression in endothelial cells								
Absent/weak	60	20.8	15.56		42	14.6	6.4	
Moderate/strong	44	20.3	18.62	0.6	38	16.6	8.7	0.37

*Mann-Whitney *U*-test, if not otherwise specified.
†Kruskal-Wallis test.
‡Seven glucagon-expressing PETs of undetermined malignant potential were added.
§Malignant tumors only.

tive subgroups or when seven additional probably malignant PETs were also included in the analysis. No association was found between VEGF-C expression and functional status (Fisher's exact test, $P = 0.07$), lymph vessel invasion (Fisher's exact test, $P = 0.16$), or lymph node metastasis (Fisher's exact test, $P = 0.39$).

Multivariate logistic regression analysis showed that moderate to strong glucagon expression ($P = 0.001$; *B*

Figure 1. a and **b:** Lymph vessels marked by podoplanin immunolabeling in normal pancreas (**a**) and in a PET (**b**). **c:** Lymph vessel invasion by tumor cells in a malignant PET (podoplanin immunostain). **d** and **e:** Double immunolabeling of proliferating lymph vessels (**arrows**) marked by chromogenic substrates for PCNA (red) and podoplanin (brown) and by fluorochromes for PCNA (red) and podoplanin (green). **f:** VEGF-C immunolabeling in a peritumoral Langerhans islet (L), note the glucagon cell pattern (**arrows**). **g:** VEGF-C expression in a PET. **h:** Fluorescent double labeling of VEGF-C (green) and glucagon (red) in a glucagon-producing PET; note the cellular co-localization (yellow). **i:** VEGF-D expression in a PET; **inset**: positive control for VEGF-D in endocrine cells of the gastric mucosa. **j:** Negative control for VEGF-D immunolabeling replacing the primary antibody with nonspecific mouse IgG2; **inset**: the same negative control in the gastric mucosa stained with control mouse immunoglobulin (see Materials and Methods). **k:** VEGF-A expression in a PET. **l:** bFGF expression in a PET, significant immunolabeling of endothelial (**arrows**) and tumor cells (T). Original magnifications: ×200 (**a**, **b**); ×400 (**i**, **j**); ×500 (**g**); ×800 (**c**, **f**, **k**, **l**); ×1000 (**d**, **e**, **h**, and **insets** in **i**, **j**).

Table 5. VEGF-C Expression Related to Clinicopathological Features

	VEGF-C, absent/weak No. of tumors	VEGF-C, moderate/strong	*P* value*
Biological behavior			
Benign all	37 (35.6%)	2 (5.9%)	
Malignant all	46 (44.2%)	19 (18.3%)	0.002
Functionally			
active	49 (47.1%)	8 (7.7%)	
inactive	34 (32.7%)	13 (12.5%)	0.07
Lymph vessel invasion‡			
absent	34 (52.3%)	11 (16.9%)	
present	12 (18.5%)	8 (12.3%)	0.16
Lymph node metastasis‡			
absent	28 (43.1%)	13 (20%)	
present	18 (27.7%)	6 (9.2%)	0.39
Hormone expression in tumor cells			
Insulin			
Absent/weak	49 (47.1%)	20 (19.2%)	
Moderate/strong	34 (32.7%)	1 (1%)	0.001
Glucagon (n = 104)			
Absent/weak	81 (77.9%)	15 (14.4%)	
Moderate/strong	2 (1.9%)	6 (5.8%)	=0.001
Absent	59 (56.7%)	5 (4.8%)	
Present	24 (23.1%)	16 (15.4%)	<0.001
Glucagon (n = 111)†			
Absent/weak	82 (73.9%)	17 (15.3%)	
Moderate/strong	3 (2.7%)	9 (8.1%)	<0.001
Absent	59 (53.2%)	5 (4.5%)	
Present	26 (23.4%)	21 (18.9%)	<0.001
Somatostatin			
Absent/weak	77 (74%)	19 (18.3%)	
Moderate/strong	6 (5.8%)	2 (1.9%)	0.508
Pancreatic polypeptide			
Absent/weak	78 (75.7%)	16 (15.5%)	
Moderate/strong	4 (3.9%)	5 (4.9%)	0.016
Gastrin			
Absent/weak	79 (76%)	21 (20.2%)	
Moderate/strong	4 (3.8%)	–	0.4

*Fisher's exact test.
†Seven glucagon-expressing PETs of undetermined malignant potential were added.
‡Malignant tumors only.

value, 28.4; 95% confidence interval, 4.1 to 194.5), moderate to strong pancreatic polypeptide expression (P = 0.007; B value, 10.2; 95% confidence interval, 1.87 to 56.2), weak glucagon expression (P = 0.029; B value, 4.4; 95% confidence interval, 1.1 to 16.8), and malignant phenotype (P = 0.052; B value, 7.1; 95% confidence interval, 1 to 52.2) were independent cause variables of VEGF-C expression, but insulin expression turned out not to be independent. Immunofluorescent double staining of glucagon and VEGF-C revealed cellular co-expression of glucagon and VEGF-C in 8 of 10 investigated PETs. However, not all VEGF-C-expressing tumor cells were positive for glucagon (Figure 1h). Low-grade VEGF-D expression was detectable in 3 of the investigated 31 PETs showing iLVD >25/0.75 mm^2, whereas the other tumors did not express VEGF-D (Figure 1, i and j).

VEGF was expressed in tumor cells (Figure 1k), in some endothelial cells and macrophages, and in islets of the peritumoral pancreas. The majority of the PETs (78 of 104, 75%) expressed VEGF in more than 10% of the tumor cells. bFGF expression was seen in the cytoplasm and nuclei of tumor cells (moderate to strong in 38 of 104 PETs) (Figure 1l). After preadsorption with specific blocking peptide no signal was detected. Endothelial cells showed strong to moderate bFGF expression in 44 of 104 tumors, as stromal cells and peritumoral fibroblasts often did.

Quantitative PCR for VEGF-C and VEGF-D mRNA Detection

RNA extracted from paraffin-embedded tissue blocks is known to be considerably degraded.[31] To estimate the degree of degradation, we compared the expression of the housekeeping gene GAPDH in six freshly cryoconserved PET tissues with that in 29 paraffin-embedded tissue blocks. The GAPDH copy number was 10 times lower in paraffin embedded tissues (mean, 1.79 attomol/100 ng; SD, 2.56 attomol/100 ng) than in snap-frozen tissue (mean, 16.7 attomol/100 ng; SD, 25.8 attomol/100 ng). This reduction of detectable mRNA copy numbers in paraffin-embedded material was lower than previously reported[31] (one-thirtieth signal intensity when paraffin-embedded material was compared with fresh tissue RNA) and indicated good RNA recovery and amplification efficacy in our assay. The application of appropriate proteinase K predigestion, usage of primers resulting in short amplicons (<140 bp), and application of a dilution series of

gel-purified external standards for the PCR reaction ensured high sensitivity and reliability of quantification.

Three independent PCR runs detecting VEGF-C mRNA were analyzed using Spearman's rank correlation analysis. The resulting correlation coefficients were 0.72, 0.8, and 0.91. The median VEGF-C mRNA expression was 13 copies/1 attomol GAPDH (mean, 2080 copies; SD, 1070; range, 0.07 to 6410 copies; mean without one outlier, 80 copies/1 attomol GAPDH). The mean mRNA levels of three PCR runs correlated with VEGF-C immunohistochemical results (mean of PCR results divided into quartiles and tested with Kendall-Tau b test, correlation coefficient 0.482, $P = 0.003$) and showed an association with iLVD (quartiles of mean iLVD compared with quartiles of mean PCR results, Kendall-Tau b test, correlation coefficient 0.355, $P = 0.023$). VEGF-D mRNA expression was below the detection limit in 27 tumors. Only in two tumors were low levels of VEGF-D measurable (0.16 to 0.84 copies/1 attomol GAPDH).

Discussion

We found that both benign and malignant PETs exhibited higher iLVD values than normal pancreata. As the strongest evidence for intratumoral lymphangiogenesis, we detected proliferating lymphatic endothelial cells in PETs with high iLVD. Angioinvasive/metastatic tumors more frequently revealed higher iLVD values than grossly infiltrating PETs. Moreover, high iLVD correlated with lymph vessel invasion in malignant PETs and with VEGF-C expression. Interestingly, VEGF-C expression was associated with glucagon and pancreatic polypeptide production in tumor cells.

For the specific immunohistochemical recognition of lymph vessels, we used an antiserum against podoplanin, a marker of lymphatic endothelial cells that was shown to co-localize with LYVE-1, a CD44-related hyaluronan receptor expressed mainly in lymphatic endothelial cells[33,34]. Apart from lymphatic endothelial cells, podoplanin was also found to be expressed in glomerular podocytes and occasionally in tumor-associated myofibroblasts.[9] In our study, the latter finding was encountered in three PETs, which were therefore excluded from further evaluation. The podoplanin antiserum identified lymph vessels in normal pancreatic tissue and in most (90%) of the PETs. The lymphatic channels encountered in the PETs were mainly small and scattered and differed markedly from the wide lymph vessels of the normal pancreas. Their density was significantly higher than in normal pancreatic tissue. Both findings suggested that the detected lymphatics in PET were newly developed vessels rather than entrapped pre-existing vessels. To find further evidence for this assumption, we used double labeling for podoplanin and the PCNA. Using this method we detected proliferating lymphatic endothelial cells in 11 of 27 PETs showing high iLVD. The proliferation rate of lymph vessels ranged between 0.7% and 3%, which can be regarded as low-grade lymphangiogenesis compared to data from angiogenesis research. Evidence for proliferating lymph vessels has so far only been reported in melanomas[3,4] and squamous cell carcinomas of the head and neck region.[1] Thus PETs represent the third human tumor type showing intratumoral lymphangiogenesis.[2]

The iLVD values detected in the 104 PETs in our series ranged from 0 to 87/0.75 mm^2. When these values were correlated with the tumors' biological behavior, it was found that they did not discriminate between benign and malignant PETs. Even small insulinomas commonly had high iLVD values. These findings suggest that intratumoral lymphangiogenesis per se may be unrelated to malignant growth. However, an association of high iLVD with aggressive behavior was found when malignant PETs were divided into ones that were grossly invasive only and ones that were angioinvasive and/or metastatic. Grossly invasive tumors infiltrated surrounding organs without showing other signs of malignancy. Although in our series they were significantly larger than angioinvasive or metastatic tumors, their iLVD values did not exceed 20/0.75 mm^2. In contrast, 26 of 58 (44.8%) angioinvasive/metastatic tumors revealed iLVD values >20 ($P = 0.022$). This may indicate that induction of lymphangiogenesis increases the angioinvasive/metastatic capacity of PETs. In line with this suggestion is the observation that tumor cells that invaded lymphatic spaces were significantly more often detected in PETs with a high iLVD than in PETs with low iLVD. This finding supports the hypothesis that an increase in tumor-related lymph vessels may contribute to tumor progression. The fact that high iLVD values were not associated with lymph node metastasis may have its explanation in the techniques used for surgical removal of the tumors. During the years in which a considerable number of the PETs in our series were collected, they did not necessarily include systematic lymphadenectomy. Alternatively, one may speculate that intratumoral lymph vessels had only limited access to the draining lymphatic system of the peritumoral tissue or that penetration of lymph vessels and the formation of lymph node metastases represent two different biological processes.

Although pLVD was increased compared to normal pancreas, pLVD was not related to iLVD, lymph vessel invasion, lymph node status, and angioinvasive/metastatic phenotype. These results were in accordance with the observation that peritumoral lymph vessel invasion was only seen in one malignant PET in our series. The findings may be explained by the expansive growth of malignant PETs, which leads to tumor demarcation and often to pseudoencapsulation, diminishing the accessibility of tumor cells to peritumoral lymphatics. In cervical carcinomas[9] high pLVD correlated with lymphatic tumor progression, while in head and neck carcinomas[1] and melanomas[3] high pLVD turned out to be a favorable prognostic factor.

Twenty percent of the PETs (21 of 104) showed moderate to strong VEGF-C expression. These immunohistochemical results were confirmed in 29 PETs by quantification of VEGF-C mRNA from formalin-fixed paraffin-embedded tumor tissue blocks using quantitative real-time RT-PCR. Although VEGF-C mRNA was

detected in virtually all PETs using this highly sensitive assay, we failed to detect VEGF-D mRNA.

PETs that exhibited moderate to strong VEGF-C expression showed a closely correlated glucagon expression of the same intensity, both in functioning (ie, glucagonomas) and nonfunctioning glucagon-expressing tumors. Even in PETs in which glucagon-expressing cells accounted for only a minor cell population of the tumor cells there was associated VEGF-C expression. In a multivariate analysis testing cause variables of VEGF-C expression, moderate to strong glucagon expression was the strongest indicator, followed by moderate to strong pancreatic polypeptide expression and weak glucagon expression. Moreover, immunofluorescent double labeling of glucagon and VEGF-C revealed cellular co-localization in 8 of 10 PETs. Because VEGF-C is also detected in the glucagon cells of the normal islets, the results in the PETs suggest that this so far unexplained relationship between VEGF-C and the normal glucagon cells is retained in neoplastic conditions. At present, there is no plausible explanation for the association between pancreatic polypeptide expression and VEGF-C expression, apart from the observation that glucagonomas frequently co-express pancreatic polypeptide.[35]

Although there is ample experimental evidence of an association between VEGF-C expression and lymphangiogenesis,[36–40] this relationship is less conspicuous in human tumors. A correlation between VEGF-C expression and high LVD was recently reported in a small series of oral squamous cell carcinomas[41] but has been shown to be absent in melanomas[3,4] and in squamous cell carcinomas of the head and neck.[1,2] In cervical carcinoma peritumoral LVD was correlated with the density of VEGF-C-expressing peritumoral macrophages.[9] Our study revealed a clear association between VEGF-C expression and high intratumoral LVD. This finding demonstrates for the first time in a large tumor series that the production of a lymphangiogenic cytokine is coupled with intratumoral lymphangiogenesis. However, statistical analysis using a multivariate regression model showed that VEGF-C (together with glucagon) accounted for high iLVD in only approximately one-fourth of the tumors (r^2 = 0.27). Because the expression of the other cytokines implicated in lymphangiogenesis, VEGF-D, bFGF, and VEGF, was not associated with iLVD, the presence of additional, possibly so far unknown, factors has to be assumed. Alternatively, posttranslational processing of known cytokines may also play a role, rendering angiogenic factors more lymphangiogenic.

In summary, we demonstrated intratumoral lymphangiogenesis in PETs. This process is, at least in part, mediated by VEGF-C expression and seems to be independent of other lymphangiogenic factors such as VEGF-D, VEGF, and bFGF. The observations that high iLVD was associated with lymph vessel invasion and with angioinvasive/metastatic tumor features indicate that lymphangiogenesis may promote the malignant progression of PETs. Our data on a large series of human PETs confirm and extend the previous reports on RipTag/RipVEGF-C transgenic mice, in which VEGF-C-induced lymphangiogenesis rendered locally invasive islet cell tumors lymphangioinvasive and metastatic.[40]

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