Enhanced Expression of Vascular Endothelial Growth Factor in Human Pancreatic Cancer Correlates with Local Disease Progression

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an angiogenic polypeptide that has been implicated in cancer growth. In the present study, we characterized VEGF expression in cultured human pancreatic cancer cell lines and determined whether the presence of VEGF in human pancreatic cancers is associated with enhanced neovascularization or altered clinicopathological characteristics. VEGF mRNA transcripts were present in all six tested cell lines (ASPC-1, CAPAN-1, MIA-PaCa-2, PANC-1, COLO-357, and T3M4). Immunoblotting with a highly specific anti-VEGF antibody revealed the presence of VEGF protein in all of the cell lines. Northern blot analysis of total RNA revealed a 5.2-fold increase in VEGF mRNA transcript in the cancer samples in comparison with the normal pancreas. Immunohistochemical and in situ hybridization analysis confirmed the expression of VEGF in the cancer cells within the tumor mass. Immunohistochemical analysis of 75 pancreatic cancer tissues revealed the presence of strong VEGF immunoreactivity in the cancer cells in 64% of the cancer tissues. The presence of VEGF in these cells was associated with increased blood vessel number, larger tumor size, and enhanced local spread but not with decreased patient survival. These findings indicate that VEGF is commonly overexpressed in human pancreatic cancers and that this factor may contribute to the angiogenic process and tumor growth in this disorder.

INTRODUCTION

Pancreatic ductal adenocarcinoma is the fifth leading cause of cancer death in the United States (1). In recent years, there has been a decrease in mortality rates and improvement in survival rates of pancreatic cancer patients following surgery such as pancreatico-duodenectomy (2-5). Nonetheless, the overall 1 year survival rate after diagnosis of pancreatic cancer is less than 20%, and the overall 5-year survival rate is only 3% (6). One reason for this poor prognosis is the propensity of pancreatic cancers to invade adjacent blood vessels and form hematogenous metastasis in the early phase of the disease, independently of primary tumor growth (7).

The growth and metastasis of cancers has been shown to be angiogenesis dependent (8), and considerable interest has developed in the possible participation of VEGF in angiogenesis. VEGF is a homodimeric glycoprotein with an approximate molecular weight of M, 46,000. It consists of four isoforms having 121, 165, 189 or 206 amino acid residues in the mature monomer. These monomers are generated by differential splicing of mRNA derived from a single gene (9, 10). All four forms are mitogenic to vascular endothelial cells and induce vascular permeabilization. Only VEGF 121 does not bind heparin, and each isoform exhibits a different affinity for heparan sulfate proteoglycans (11). Two related transmembrane receptors bind VEGF with high affinity. Both are class III transmembrane protein tyrosine kinases. VEGF receptor-1 was originally named the fms-like tyrosine kinase, and is also known as flt (12). VEGF receptor-2, also known as KDR, is the human homologue of flik-1 (13).

VEGF and its receptors play an important role in angiogenesis during embryonic development and wound healing (14, 15). A number of studies have suggested that VEGF may have an important role in tumor growth and metastasis (16-18). It is not known, however, whether VEGF has a role in human pancreatic cancer. Therefore, in the present study, we examined the expression of VEGF in cultured pancreatic cancer cell lines and in surgical specimens from patients with pancreatic cancer. We now report that VEGF is expressed in pancreatic cancer cell lines and in human pancreatic cancers.

MATERIALS AND METHODS

Materials. The following were purchased: DMEM, RPMI 1640, fetal bovine serum, trypsin-EDTA solution, and...
penicillin streptomycin solution from Irvine Scientific (Santa Ana, CA); gene screen membranes from New England Nuclear (Cleveland OH); random-primed labeling kit, digoxigenin RNA labeling kit, and digoxigenin nucleic acid detection kit from Boehringer-Mannheim (Indianapolis, IN); [α-32p]dCTP (3000 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); a highly specific VEGF polyclonal antibody from Santa Cruz Biotech (Santa Cruz, CA); a highly specific anti-factor VIII mouse monoclonal antibody from DAKO Corp. (Carpinteria, CA); leupeptin from United States Biochemical Corp. (Cleveland, Ohio); Immobilon P membrane from Millipore Intertiect (Bedford, MA); and Hyperfilm-ECL from Amersham. The VEGF 165 cDNA (19) was a gift from Dr. Judith A. Abraham (Mountainview, CA). All other chemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Cell Culture. ASPC-1, CAPAN-1, Mia-PaCa-2, and Panc-1 human pancreatic cancer cells were obtained from the American Type Culture Collection (Rockville, MD). T3M4 and COLO-357 human pancreatic cancer cells were a gift from R. S. Metzger at Duke University. Cells were grown in monolayer culture in a humidified 5% CO2 and 95% air atmosphere at 37°C. COLO-357, CAPAN-1, and T3M4 cells were grown in RPMI 1640. COLO-357, Mia-PaCa-2, and Panc-1 cells were grown in DMEM. Media contained antibiotics and 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Tissues for Northern Blot Analysis. Normal pancreatic tissue samples were obtained from 10 healthy individuals (7 males and 3 females; mean age, 40.5 ± 15.6 years; range, 22–64 years) through an organ donor program. Pancreatic cancer tissue samples were obtained from 15 patients (7 males and 8 females; mean age, 60.2 ± 12.3 years; range, 32–78 years) who underwent surgery for pancreatic cancer. Tissue samples were frozen in liquid nitrogen and held at −80°C until use for RNA extraction.

Tissues for Immunohistochemistry. Normal pancreatic tissue samples were obtained from four male and one female donors, through an organ donor program. The age of the organ donors ranged from 47 to 65 years, with a mean age of 55.6 ± 6.6 years. Pancreatic carcinoma tissue samples were obtained from 75 patients (45 males and 30 females; mean age, 62.4 ± 9.6 years; range, 31–77 years) undergoing surgery for pancreatic cancer. The vast majority of these patients (66 individuals) did not have gross evidence for metastatic disease at the time of clinical presentation. The tumors were classified according to the Tumor-Node-Metastasis classification for pancreatic cancer (20). Histologically, there were 13 grade 1, 44 grade 2, 17 grade 3, and 1 grade 4 ductal adenocarcinomas. There were 15 stage I, 9 stage II, 42 stage III, and 9 stage IV tumors. Tissues were fixed in Bouin’s solution or 10% paraformaldehyde solution for 18–20 h and embedded in paraffin. All studies were approved by the Human Subjects Committees of the University of California (Irvine, CA), the University of Bern (Bern, Switzerland), and the Ethics Committee of the Yamanashi Medical University (Yamanashi, Japan).

Northern Blot Analysis. Total RNA was extracted by the acid guanidinium thiocyanate method, and poly(A) + mRNA was prepared by oligodeoxythymidine column chromatography (21). RNA was fractionated on 0.8% agarose/2.2% formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation (22). The blots were prehybridized and hybridized with the indicated α-32P-labeled cDNA probes and washed under high stringency conditions as described previously (22). For use in hybridizations, the VEGF cDNA was subcloned into the pLen eukaryotic expression vector using a BamHI restriction site (19). Equivalent loading of RNA in each lane was confirmed by hybridizing the total RNA filters with a mouse 7S cDNA that cross-hybridizes with human cytoplasmic 7S RNA. Equivalent loading of poly(A) + mRNA filters was confirmed by hybridization with a glyceraldehyde phosphate dehydrogenase cDNA (23). All cDNAs were labeled with [α-32p]dCTP using a random primer labeling system (24). Blots were exposed at −80°C to Kodak XAR-5 film using intensifying screens. Densitometric analysis of the autoradiograms was performed with a LKB Ultrascan XL enhanced laser densitometer (Upsala, Sweden).

Immunohistochemistry. Human pancreatic cancer cells were solubilized in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 1 mM sodium vanadate, 50 mM sodium fluoride, 2 mM EDTA (pH 8.0), 100 μg/ml benzamidine, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Proteins were subjected to SDS-PAGE and transferred to Immobilon P membranes. Membranes were incubated for 90 min with a highly specific anti-VEGF polyclonal antibody that recognizes VEGF121, VEGF165, and VEGF189. This antibody was raised against a glutathione S-transferase fusion protein construct containing human VEGF sequences corresponding to amino acids 1–191 with 44 amino acid detection from amino acids 142–185 (25). Membranes were washed and incubated with a horseradish peroxidase-coupled secondary goat anti-rabbit antibody for 60 min. After washing, antibodies were visualized by enhanced chemiluminescence.

Immunohistochemistry. The same anti-VEGF polyclonal antibody that was used for immunoblotting was also used for immunohistochemical analysis. This antibody was shown previously to be highly specific in immunohistochemical reactions of human gastric and lung cancers (26, 27). Paraffin-embedded sections (4 μm) from pancreatic cancer and normal pancreatic tissues were subjected to immunostaining using the streptavidin-peroxidase technique. After deparaffinization, endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 min (23°C) with 10% normal goat serum and then incubated with polyclonal VEGF antibody (0.03 mg/ml in PBS containing 1% BSA) for 16 h at 4°C. Bound antibody was detected with biotinylated goat anti-rabbit IgG secondary antibody and streptavidin-peroxidase complex (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer’s hematoxylin. Some sections were incubated with nonimmunized rabbit IgG or without primary antibodies, which did not yield positive immunoreactivity. Scoring was carried out by two independent observers blinded to the patient’s status. Positive staining was defined as the presence of VEGF immunoreactivity in at least 10% of the cancer cells. A factor VIII mouse monoclonal antibody (1:100 dilution) was used to stain endothelial cells. The number of capillaries and microvessels adjacent to the foci of cancer cells was determined...
at \times 100 after identifying highly vascular areas at \times 40 (27). Both evaluators were blinded with respect to patient’s history and immunohistochemical results.

**In Situ Hybridization Analysis.** To carry out in situ hybridization analysis, tissue sections (4-μm thick) were placed on 3-aminopropylmethoxysilane-coated slides, deparaffinized, and incubated at 23°C for 20 min with 0.2 N HCl and at 37°C for 15 min with 20 μg/ml proteinase K. The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde, incubated briefly twice with PBS containing 2 mg/ml glycine and once for 1 h in 50% (v/v) formamide/2X SSC. (1 X SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0.) The hybridization buffer contained 0.6 μM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 μg/ml yeast tRNA, 1 X Denhart’s solution, 10% dextran sulfate, 40% formamide, and 500 ng/ml of the indicated digoxigenin-labeled riboprobe. Hybridization was performed in a moist chamber for 16 h at 42°C. The sections were then washed sequentially with 50% formamide/2X SSC for 30 min at 50°C, 2X SSC for 20 min at 50°C, and digested with RNase A (1 μg/ml) in TNE solution (10 mM Tris-HCl, 500 mM NaCl, and 1 mM EDTA, pH 7.6) for 15 min at 37°C. The sections were washed with TNE solution for 10 min at 37°C and 0.2X SSC for 20 min at 50°C.

For immunological detection, the Genius 3 nonradioactive nucleic acid detection kit was used. The sections were washed briefly with buffer 1 solution (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated for 60 min at 23°C with 1% (w/v) blocking reagents in buffer 1. The sections were then incubated for 30 min at 23°C with a 1:2000 dilution of alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween 20. The sections were then washed twice for 15 min at 23°C in buffer 1 solution containing 0.2% Tween 20 and equilibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂, pH 9.5) for 2 min. The sections were then incubated with color solution containing nitroblue tetrazolium and X-phosphate in a dark box for 2-3 h. After the reaction was stopped with TE buffer (10 msi Tris-HCl, 100 mM NaCl, 1 msi EDTA, pH 9.5), the sections were dehydrated and mounted in aqueous mounting medium.

**Statistical Analysis.** Differences in distribution of VEGF were determined using the χ² exact test. Kaplan-Meier survival analysis was used to estimate survival time, and log-rank test was used to compare differences in survival time between VEGF-positive and VEGF-negative groups (28, 29). For all tests, P < 0.05 was considered to be significant.

**RESULTS**

Analysis of poly(A)⁺ RNA isolated from human pancreatic cancer cell lines indicated that COLO-357, T3M4, ASPC-1, and CAPAN-1 cells expressed the 4.1-kb VEGF transcript, whereas MIA-PaCa-2 and PANC-1 cells expressed the 3.3-kb VEGF transcript. In this cell line, there was also a faint 1.8-kb transcript (Fig. 1). Immunoblotting with a highly specific anti-VEGF-antibody revealed an approximately Mf, 43,000 band in all of the cell lines and occasionally also a Mf, 41,000 band (Fig. 2), both of which correspond to the VEGF165 isoform homodimer. In addition, Mf, 32,000 and Mf, 31,000 bands corresponding to the VEGF121 isoform homodimer were seen in MIA-PaCa-2 cells and, to a lesser extent on the original immunoblot, in PANC-1 cells (Fig. 2). Highest levels of VEGF mRNA and protein were observed in T3M4 and ASPC-1 cells (Figs. 1 and 2).

Northern blot analysis of total RNA isolated from human pancreas revealed the presence of the 4.1-kb VEGF transcript in all normal pancreatic samples and the cancer samples (Fig. 3). Densitometric analysis of the autoradiograms indicated that the levels of this 4.1-kb transcript were 5.2-fold higher in the pancreatic cancers by comparison with the normal pancreas, and this difference was statistically significant (P < 0.05). In two cancer samples (Fig. 3, Lanes 7 and 8), a 3.3-kb VEGF transcript was also visible. In addition, in one cancer sample an approximately 4.6-kb transcript was also evident (data not shown).

The same highly specific anti-VEGF antibody that was used in the immunoblotting studies was used next to localize VEGF immunohistochemically. In the normal pancreas, moderate to strong VEGF immunoreactivity was present in the cytoplasm of endocrine islet cells, in some ductal cells within the small ductules, and in a few acinar cells (Fig. 4A). In the pancreatic cancers, moderate to strong VEGF immunoreactivity was present in the cytoplasm of many of the cancer cells (Fig. 4B) in 4B (64%) of the 75 cancers. Occasionally, the cancer cells also exhibited strong apical VEGF immunoreactivity (Fig. 4B). Faint VEGF immunoreactivity was also present in some of the fibroblasts within the connective tissue around cancer cells (Fig. 4B) and moderately intense VEGF immunoreactivity was also seen in vascular smooth muscle cells of blood vessels (data not shown).
**Fig. 2** Immunoblot analysis of VEGF expression in human pancreatic cancer cell lines. Cell lysates (50 μg/lane) were prepared from COLO-357 (Lane 1), MIA-PaCa-2 (Lane 2), PANC-1 (Lane 3), T3M4 (Lane 4), ASPC-1 (Lane 5), and CAPAN-1 (Lane 6) cells, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblotting with a highly specific anti-VEGF antibody. Left: migration positions of molecular weight markers (in thousands).

**Fig. 3** Northern blot analysis of VEGF expression in human pancreatic tissues. Total RNA (20 μg/lane) was isolated from four normal (Lanes 1–4) and seven pancreatic cancer tissues (Lanes 5–11), size-fractionated, and hybridized with 32P-labeled VEGF cDNA (700,000 cpm/ml; 7 days of exposure) and 7S cDNA (30,000 cpm/ml; 6 h of exposure). Left: migration positions of 28S and 18S ribosomal subunits.

**Fig. 4** VEGF immunoreactivity in the human pancreas. In the normal pancreas (A), VEGF immunoreactivity was always present in the islets (arrowheads) and occasionally present in the ductal cells of small ductules and in a few acinar cells. In the pancreatic cancers (B), the ductal-like cancer cells exhibited abundant VEGF immunoreactivity in the cytoplasm. Occasionally, these cells exhibited apical VEGF immunoreactivity (arrows) in addition to cytoplasmic immunoreactivity. A and B, ×500.

To confirm the immunostaining results, in situ hybridization analysis was next carried out in the cancer tissues. Foci of cancer cells that were positive for VEGF immunoreactivity (Fig. 5, A and B) also exhibited a specific mRNA in situ hybridization signal (Fig. 5C). A faint to moderate VEGF in situ hybridization signal was also present in some of the fibroblasts around cancer cells (Fig. 5C), as well as in the islet cells surrounding the cancer areas (data not shown). Treatment of the sections with excess RNase abolished these in situ hybridization signals (Fig. 5D).

**DISCUSSION**

Due to alternative splicing of mRNA, VEGF has four different mature isoforms: VEGF121, VEGF165, VEGF189, and VEGF206 (10, 19). VEGF165 is the most abundant form in the majority of cells and tissues (10, 19). All four isoforms are mitogenic toward endothelial cells (30). The two larger isoforms, VEGF189 and VEGF206, have a high affinity toward cancer cells was associated with a statistically significant increase in tumor size and local extension (T category). However, there was no correlation between the presence of VEGF in the cancer cells and the histological grade or tumor stage of the cancers (Table 1). The 30-month survival of the VEGF-negative and VEGF-positive groups were 7.4 and 2.1%, respectively, and the mean survival duration of these two groups was 14.8 ± 13.4 and 10.8 ± 10.3 months, respectively. Thus, there was a tendency for shorter survival in the patients with VEGF-positive tumors. However, Kaplan-Meier analysis (Fig. 7) and the log-rank test indicated that there was no significant difference in survival between these two groups.
heparin and bind to the extracellular matrix following their release from their cells of origin (9, 31). In contrast, the two shorter isoforms, VEGF121 and VEGF165, have low or no affinity to heparin and are secreted as diffusible molecules (9, 31). Following incorporation into the extracellular matrix, VEGF165, VEGF189, and VEGF206 are released from their bound states by proteolysis, and the released dimers have same activity as VEGF121 (9, 31). Therefore, it has been suggested that the bioavailability of VEGF may be regulated by mechanisms that control alternative splicing, which than dictates whether VEGF will be soluble or incorporated into a biological reservoir (30).

Previous studies have demonstrated that colorectal and breast cancer cell lines express relatively high levels of VEGF mRNA (16, 32, 33) and that ovarian cancer cell lines express high levels of VEGF as determined by immunoblotting (18). In the present study, we determined that cultured human pancreatic cancer cell lines also express relatively high levels of VEGF. Four of the six tested cell lines expressed the 4.1-kb VEGF transcript that encodes VEGF189, whereas the remaining two cell lines (MIA-PaCa-2 and PANC-1) expressed the 3.3-kb VEGF transcript that encodes VEGF165. T3M4 cells also expressed a 1.8-kb transcript of unknown significance. In general, there was a good correlation between VEGF mRNA expression in these cell lines and VEGF protein levels, as determined by immunoblotting. All of the cell lines expressed the Mr 43,000 VEGF proteins corresponding to the VEGF165 isoform ho-
Table 1  VEGF expression in relation to the clinicopathological characteristics of 75 patients with pancreatic cancer

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* The rates of VEGF immunoreactivity (positive or negative) in different patient subgroups were compared by the χ² two-sided exact trend test. NS, not significant.

* TNM, Tumor-Node-Metastasis.

modimer. The two cell lines that expressed the shorter 3.3-kb VEGF transcript also exhibited the smaller M₄, 32,000 and M₃, 31,000 protein bands corresponding to the VEGF121 isomorph homodimer. These observations confirm the specificity of the anti-VEGF antibody used in the present study and indicate that human pancreatic cancer cells express VEGF at the mRNA and protein levels, and that in some instances they express more than one VEGF isoform.

Several lines of evidence in the present study point toward aberrant VEGF expression in pancreatic cancers in vivo: (a) Northern blot analysis revealed the presence of a single 4.1-kb VEGF mRNA transcript in all normal pancreatic tissues and in all pancreatic cancer samples. However, 2 of the 15 cancer samples expressed a 3.3-kb VEGF mRNA transcript, and 1 cancer sample exhibited an approximately 4.6-kb VEGF mRNA transcript. The presence of a multiple VEGF mRNA transcripts is consistent with the propensity of cancer tissues to express different VEGF isoforms (16, 32, 33, 34–37) and with our findings in the pancreatic cancer cell lines; (b) Northern blot analysis revealed a 5.2-fold increase in the 4.1-kb VEGF mRNA transcript in the cancer samples by comparison with the normal samples, indicating that VEGF is overexpressed in pancreatic cancers; and (c) the distribution of VEGF in the normal and cancerous tissues was different. Thus, in the normal pancreas, VEGF was relatively abundant in the endocrine islets, less frequently present in the ductal cells, and only occasionally present in acinar cells. In contrast, in many of the pancreatic cancers, VEGF was abundant in the ductal-like cancer cells. Inasmuch as VEGF is a specific mitogen toward endothelial cells, these observations suggest that various isoforms of cancer cell-derived VEGF have the potential to act in a paracrine manner on endothelial cells within the pancreatic tumor mass, thereby leading to enhanced angiogenesis and greater tumor growth.

Overexpression of VEGF has been reported in brain, mammary, colorectal, renal, liver, ovarian, and gastric malignancies (16, 17, 32, 33, 34–36, 38, 39). In the present study, 64% of the pancreatic cancer samples exhibited VEGF immunoreactivity in the cancer cells within the tumor mass. The reasons for this overexpression are not known. However, it has been demonstrated that mutations in the H-ras or K-ras oncogene are associated with marked up-regulation of VEGF (33), and K-ras mutations are frequent in pancreatic cancer (40–42). Furthermore, VEGF expression is enhanced by epidermal growth factor, PDGF-BB, and TGF-β isoforms (43–46), and the epidermal growth factor family of ligands, TGF-β isoforms, and PDGF and its receptors are overexpressed in this malignancy (22, 47–50). Together, these alterations may combine to enhance VEGF expression in pancreatic cancer.

Univariate analysis of the immunohistochemical data demonstrated that the presence of VEGF in the pancreatic cancer cells was associated with enhanced tumor size and extension and greater neovascularization, indicating that VEGF has the potential to contribute to pancreatic tumor growth in vivo. Although there was a tendency for shorter survival in the group with VEGF-positive tumors, this correlation was not statistically significant. Because the vast majority of the patients in the present study did not have detectable metastatic disease at the time of presentation for surgery, it cannot be determined from the limited number of cases with metastases whether VEGF expression correlates with enhanced propensity of the pancreatic cancer to metastasize. In addition, the present study did not take
into account the angiogenic potential of other growth factors that are overexpressed in pancreatic cancer, including basic fibroblast growth factor, PDGF-BB, TGF-α, hepatocyte growth factor, and TGF-β (22, 51–54). Additional studies are, therefore, necessary to determine whether inclusion of a larger number of patients or consideration of the role of other angiogenic factors may reveal a significant correlation between VEGF expression, the expression of other angiogenic factors, and decreased survival.

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