Vascular Endothelial Growth Factor-Trap Suppresses Tumorigenicity of Multiple Pancreatic Cancer Cell Lines

Mitsuharu Fukasawa and Murray Korc
Departments of Medicine, and Pharmacology and Toxicology, Dartmouth Medical School and Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire

ABSTRACT

Purpose: Vascular endothelial growth factor A (VEGF-A) is a potent angiogenic agent that binds to two high affinity VEGF receptors (VEGFRs), a process facilitated by the low affinity neuropilin receptors. Although VEGF-A is overexpressed in pancreatic ductal adenocarcinoma, it is not known whether the in vivo growth of multiple pancreatic cancer cells can be efficiently blocked by VEGF-A sequestration.

Experimental Design: Four human pancreatic cancer cell lines were grown s.c. in athymic nude mice. One cell line also was used to generate an orthotopic model of metastatic pancreatic cancer. The consequences of VEGF-A sequestration on tumor growth and metastasis were examined by injecting the mice with a soluble VEGFR chimer (VEGF-Trap) that binds VEGF-A with high affinity.

Results: VEGF-Trap, initiated 2 days after tumor cell inoculation, suppressed the s.c. growth of four pancreatic cancer cell lines and markedly decreased tumor microvessel density. In addition, using an orthotopic model of PDAC to assess the consequences of blocking VEGF-A in angiogenesis in PDAC, we used an s.c. nude mouse model of PDAC to assess the consequences of blocking VEGF-A action with VEGF-Trap, a modified soluble VEGFR that consists of the second immunoglobulin-like domain of VEGFR-1 and the third immunoglobulin-like domain of VEGFR-2 (17). We report that VEGF-Trap suppresses the s.c. growth of four distinct pancreatic cancer cell lines in athymic nude mice and that this effect is associated with a marked decrease in microvessel density. In addition, using an orthotopic

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is responsible for >20% of deaths caused by gastrointestinal malignancies, making it the fourth most common cause of cancer-related mortality in the United States and other industrialized countries. The prognosis of patients with PDAC is extremely poor, with overall 5-year survival rates <1% (1), 1-year overall survival of 12%, and a median survival of 6 months (2). Survival often is limited to patients who had surgical resection at an early stage of the disease. However, the diagnosis of PDAC often is established at an advanced stage, precluding patients from undergoing tumor resection. Despite recent therapeutic advances (3–5), these statistics have remained dismal because of the tumor’s propensity to metastasize when small and undetectable, the advanced stage at which many patients first develop symptoms, and the intrinsic resistance of pancreatic cancer cells to cytotoxic agents and/or radiotherapy (3–5).

Although PDAC is not a grossly vascular tumor, this malignancy often exhibits enhanced foci of endothelial cell proliferation and frequently overexpresses vascular endothelial growth factor (VEGF), a potent angiogenic factor that is secreted by many tumor cell lines (6). The principal form of VEGF is a homodimeric glycoprotein that has been renamed VEGF-A. It consists of five major isoforms with 121, 145, 165, 189, and 206 amino acid residues, respectively, that originate as a result of alternative splicing from a single gene (7–10). All of the five isoforms are mitogenic toward vascular endothelial cells and act by binding to two related tyrosine kinase receptors, VEGFR-1 and VEGFR-2, on the surface of endothelial cells (11–13). A third high affinity VEGF receptor, VEGFR-3, is expressed in lymphatic vessels (14–15). The three VEGFRs are transmembrane protein tyrosine kinases that possess seven immunoglobulin-like sequences in their extracellular domains and a kinase insert in their intracellular domains.

PDACs overexpress multiple additional angiogenic growth factors, including epidermal growth factor; transforming growth factor β isoforms; hepatocyte growth factor; fibroblast growth factors (FGFs) such as FGF-1, FGF-2, and FGF-5; and platelet-derived growth factor (16). Therefore, it has not been firmly established that VEGF-A is of crucial importance in promoting the angiogenic process in PDAC. To address the potential role of VEGF-A in angiogenesis in PDAC, we used an s.c. nude mouse model of PDAC to assess the consequences of blocking VEGF-A action with VEGF-Trap, a modified soluble VEGFR that consists of the second immunoglobulin-like domain of VEGFR-1 and the third immunoglobulin-like domain of VEGFR-2 (17). We report that VEGF-Trap suppresses the s.c. growth of four distinct pancreatic cancer cell lines in athymic nude mice and that this effect is associated with a marked decrease in microvessel density. In addition, using an orthotopic
model, VEGF-Trap is shown to attenuate intrapancreatic tumor growth and regional and distant metastasis. These findings support the hypothesis that VEGF-A has an important role in pancreatic cancer in vivo and raise the possibility that VEGF-Trap may ultimately provide a novel therapeutic option for management of this disease.

MATERIALS AND METHODS

Materials. The following were purchased: DMEM, RPMI 1640, fetal bovine serum, trypsin-EDTA, and glutamine-penicillin-streptomycin from Irvine Scientific (Santa Ana, CA); BxPC3 and PANC-1 human pancreatic cancer cell lines from American Type Culture Collection (Manassas, VA); and oligonucleotide primers for quantitative PCR from Applied Biosystems (Foster City, CA). The following were gifts: T3M4 and COLO-357 from Dr. R. S. Metzger at Duke University and Chimeric VEGF-Trap protein from Regeneron Pharmaceuticals (Tarrytown, NJ).

Cell Culture. Human pancreatic cancer cells were maintained in monolayer culture at 37°C in a humidified incubator with 5% CO₂/95% air. BxPC3 and T3M4 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (complete RPMI), whereas COLO-357 and PANC-1 were grown in DMEM that was similarly supplemented (complete DMEM). These cell lines release variable levels of VEGF-A into conditioned medium, which range from intermediate (PANC-1) to relatively high (BxPC3) levels (18). They also harbor K-ras, p53, and/or Smad4 mutations or deletions and therefore are highly representative of cancer cells in PDAC (19–21).

In Vivo Tumorigenicity Assay. The effects of VEGF-Trap on tumor formation and growth were assessed for all of the four cell lines using an s.c. nude mouse model. One million cells per cell line were injected s.c. at one site in the flank region of four cell lines using an s.c. nude mouse model. One million cells Trap on tumor formation and growth were assessed for all of the –(BxPC3) levels (18). They also harbor K-ras, p53, and/or Smad4 mutations or deletions and therefore are highly representative of cancer cells in PDAC (19–21).

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T3M4 cells (Fig. 1). By contrast, the T3M4-derived tumors from control animals were large and started to ulcerate, necessitating termination of these mice (Fig. 1). By the third week, there was a significant difference in tumor growth with COLO-357 cells. Thus, the growth of tumors in the VEGF-Trap group was markedly blunted, whereas tumors from control animals exhibited relatively rapid growth necessitating termination of the mice at week 5 (Fig. 1). Tumors formed by BxPC3 and PANC-1 cells exhibited a significant difference between control mice and VEGF-Trap mice by week 4, which persisted throughout the 6 weeks of the experiments (Fig. 1). Overall, by comparison of control mice with mice injected with vehicle only, there was 97% inhibition of tumor growth with COLO-357 cells (5 weeks) and PANC-1 cells (6 weeks), 92% inhibition with BxPC-3 cells (6 weeks), and 89% inhibition with T3M4 cells (2 weeks).

Effects of VEGF-Trap on Tumor Angiogenesis. To evaluate the effects of VEGF-Trap on tumor-associated angiogenesis, the tumors from the aforementioned studies were immunostained with anti-CD31 antibodies to delineate the presence of endothelial cells. The tumors from mice treated with control buffer exhibited numerous endothelial cells throughout the tumor mass (Fig. 2A). By contrast, tumors from mice treated with VEGF-Trap exhibited a marked decrease in CD31 immunoreactivity, and regions containing scant cellular material (Fig. 2B), suggesting that either necrosis or apoptosis of the cancer cells might have occurred in these areas. Moreover, quantitative morphometry with the Image-Pro Plus image analysis system revealed that the mean microvessel density (CD31-positive regions) was markedly decreased in tumors treated with VEGF-Trap compared with control tumors in all of the four cell lines (Fig. 3).

Effects of VEGF-Trap on the Expression of VEGF Receptors and Ligands. In view of the marked decrease in microvessel density in the tumors of VEGF-Trap-treated mice, we next sought to determine whether VEGF-Trap altered the expression of VEGF receptors or ligands in the tumors. Because of the limited amount of material (small tumor size) that was available for analysis in the VEGF-Trap-treated group, ligand and receptor expression was only examined in tumors from T3M4 cells. Q-PCR of tumor RNA revealed that both groups of tumors expressed relatively high VEGF-A levels, moderate VEGF-B and VEGF-C levels, and relatively low VEGF-D levels (Fig. 4A). Moreover, VEGF-Trap treatment did not significantly alter the levels of any of these mRNA moieties (Fig. 4A).

Detectable levels of VEGFR-1 and VEGFR-3 also were present in tumors from both groups, whereas neuropilin-1 and -2 mRNA levels were relatively high in both groups, and VEGFR-2 mRNA levels were below the level of detection (Fig. 4B). In contrast to the lack of an effect with respect to ligand expression, VEGF-Trap injections were associated with small but significant decreases in the expression of VEGFR-1 and neuropilin-1 and -2 mRNA levels, whereas the levels of VEGFR-3 were similar in both groups (Fig. 4B).

Fig. 1  Effects of vascular endothelial growth factor (VEGF)-Trap on in vivo tumorigenicity of pancreatic cancer cells. BxPC3, COLO-357, PANC-1, and T3M4 cells (1 × 10^6 cells/site) were injected s.c. in athymic nude mice. Two days later, twice-weekly s.c. injections (nape of the neck) of VEGF-Trap protein (or control buffer) were initiated, using a dose of 25 mg/kg, and continued for 2–6 weeks. Tumor volumes (in mm^3) were calculated as described in “Materials and Methods” and expressed as mean ± SE. *, P < 0.05; **, P < 0.01 when compared with respective controls.

Fig. 2  CD31 immunoreactivity in tumors formed by COLO-357 cells. Immunohistochemical staining for CD31 was performed as described in “Materials and Methods.” A, tumor from a control mouse. B, tumor from a mouse treated with vascular endothelial growth factor-Trap. Open arrows denote areas of scant cellular content in the central portion of the small tumor. Original magnification, ×100.
Effect of VEGF-Trap on Tumor Growth and Metastasis in an Orthotopic Model. The s.c. growing PANC-1-derived tumors were implanted into the pancreas of nude mice. Nine weeks following tumor implantation, six of six control mice had large intrapancreatic tumors with extensive local lymph node enlargement and mesenteric lymph node metastasis (Table 1). Five of these mice exhibited peritoneal dissemination, and two mice had ascites. By contrast, the intrapancreatic tumors in the six VEGF-Trap-treated mice were significantly smaller (Table 1). Moreover, one mouse did not exhibit any lymph node enlargement, and five mice exhibited local lymph node enlargement, but these nodes were significantly fewer in number (Table 1) and visibly smaller than in the control group. Only two of the VEGF-Trap-treated mice had mesenteric lymph node metastasis, and only one mouse had peritoneal dissemination (Table 1). None of the VEGF-Trap-treated mice had ascites.

DISCUSSION
Angiogenesis is believed to be essential for growth and metastasis of solid malignancies, and most (26–28), but not all (29), of the studies have reported a positive correlation between tumor VEGF-A levels, blood vessel density, and disease progression in PDAC. Moreover, VEGF-A expression in pancreatic PDAC may be associated with enhanced local spread and liver metastasis and decreased patient survival (26–28). Two additional types of studies suggest that VEGF-A may have an important role in PDAC. In vitro, pancreatic cancer cells secrete biologically active VEGF-A, which is the major angiogenic agent produced by these cells (18). In vivo, antiangiogenic therapy is effective at suppressing tumor growth in animal models of pancreatic cancer. Thus, the antiangiogenic agent TNP-470 reduces angiogenesis in tumors formed by pancreatic cancer cells, thereby decreasing their growth and metastasis (30); suppression of VEGF-A expression with a VEGF anti-sense construct attenuates tumorigenicity in nude mice (18); and adenoviral vectors carrying sequences encoding soluble VEGFR-1 and VEGFR-2 (31–32) or the VEGFR tyrosine kinase inhibitor PTK 787 (33) inhibit the growth and/or metastasis of pancreatic cancer cell tumors in mice. Together, these observations suggest that VEGF-A may have an important role in PDAC.

Soluble forms of VEGFR-1 generally exhibit nonspecific interactions with extracellular matrix and poor pharmacokinetics (17, 34). By contrast, VEGF-Trap, which was created by fusing the second immunoglobulin domain of VEGFR-1 with the third immunoglobulin domain of VEGFR-2, has minimal interactions with the extracellular matrix, an excellent pharmacokinetic profile, and a high affinity for VEGF-A with a $k_d$ that is in the ps range (17). These are important characteristics in PDAC because this cancer often exhibits intense desmoplasia and the fibroblasts within this rich extracellular matrix also express high levels of VEGF-A (25).

In the present study we determined that administration of VEGF-Trap markedly suppressed the s.c. growth of four distinct human pancreatic cancer cell lines in athymic nude mice. Moreover, this growth suppression was associated with a marked decrease in microvessel density. Inasmuch as VEGF-Trap did not alter the expression of either VEGF-A or related moieties, our findings indicate that VEGF-Trap interfered with angiogen-
following VEGF-Trap administration was reported previously with were not altered by VEGF-Trap. Decreased VEGFR-2 expression levels were below the level of detection, whereas VEGFR-3 levels in vivo and that have been implicated in promoting angiogenesis. Moreover, the present study we determined that a relatively low dose of VEGF-Trap (10 mg/kg) initiated 3 weeks following tumor implantation induced a significant reduction of the mean intrapancreatic tumor volume compared with the mean tumor volume in control mice, and a marked decrease in metastatic frequency. Together, these observations suggest that VEGF-Trap may be useful to manage established tumors and their metastases. The present findings also are noteworthy in the context of the clinical course of PDAC, which is characterized by an early propensity to metastasize and a high risk for disease recurrence following resection. The marked overexpression of VEGF-A in PDAC (25), its ability to act as a survival factor for endothelial cells and perivascular cells and to render endothelial cells more radioresistant (47), and its capacity to promote cancer cell survival (43, 48–50) and suppress cancer-directed immune mechanisms (51) suggest that a VEGF-Trap-based strategy designed to sequester VEGF-A and block its actions in PDAC may have a unique therapeutic benefit for PDAC patients who have unresectable tumors and for patients who have undergone resection and who are at high risk for disease recurrence.

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