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Pathways for aberrant angiogenesis in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease. Although the specific mechanisms that dictate its biological aggressiveness are not clearly established, it is characterized by a variety of molecular alterations as well as by the overexpression of mitogenic and angiogenic growth factors and their receptors. PDACs also express high levels of vascular endothelial growth factor (VEGF). Recent studies indicate that suppression of VEGF expression attenuates pancreatic cancer cell tumorigenicity in a nude mouse model, and that VEGF can exert direct mitogenic effects on some pancreatic cancer cells. These findings suggest that cancer cell derived VEGF promotes pancreatic cancer growth in vivo via a paracrine angiogenic pathway and an autocrine mitogenic pathway, and provide novel opportunities for therapeutic intervention in this deadly disease.

Carcinoma of the pancreas: An overview

Pancreatic ductal adenocarcinoma (PDAC) is responsible for over 20% of deaths due to 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 that are less than 1% [1], one-year overall survival of 12%, and a median survival of 6 months [2]. Survival is often limited to patients who had surgical resection at an early stage of the disease. However, the diagnosis of PDAC is often established at an advanced stage, precluding patients from undergoing tumor resection in spite of limited results with other treatment modalities [3]. These dismal statistics are due to 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 radiotherapy [3–5]. PDAC may be an even more serious problem in the future since its incidence increases after age 50 and the general population world-wide is aging. There is, therefore, an urgent need for an improved understanding of the mechanisms that contribute to pancreatic tumor growth and metastasis, and for the design of therapies for this disorder that are more effective than current regimens. This review will cover in a brief manner the molecular biology of pancreatic cancer, and will then focus on various aspects of vascular endothelial growth factors in angiogenesis in general and in relation to PDAC in particular.

Molecular biology of pancreatic cancer

A plethora of genetic mutations have been described in the cancer cells of PDAC patients. The most frequent alterations (approximate frequency indicated in parenthesis) include mutations in the K-*ras* oncogene (90%), the p53 (85%) and Smad4 (50%) tumor suppressor genes, and the p16 (85% mutated and 15% silenced epigenetically) cell cycle inhibitory gene [6,7]. Together, these alterations promote cellular proliferation, suppress apoptotic pathways, and facilitate tumor spread and metastasis. In addition, there is overexpression of multiple tyrosine kinase

receptors and their ligands which enhances mitogenesis, and loss of responsiveness to the growth-inhibitory signals of members of the transforming growth factor beta (TGF-β) family [6,7], which contribute in a significant manner to the biological aggressiveness of PDAC.

It is well established that human pancreatic cancer cell lines overexpress the epidermal growth factor (EGF) receptor (EGFR) and produce multiple ligands that bind directly to EGFR, including transforming growth factor-alpha (TGF-α, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin and epiregulin [8–12]. These cell lines also express other growth factors such as fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) B chain [13–16]. However, expression of receptors and ligands in cell lines does not necessarily indicate parallel alterations in PDAC *in vivo*. Therefore, studies using human tissues have been of vital importance in this regard. Studies using immunohistochemistry, Northern blot analysis and *in situ* hybridization techniques, have demonstrated that PDAC tissue samples overexpress EGFR and six ligands that bind directly to EGFR (EGF, TGF-α, HB-EGF, betacellulin, epiregulin and amphiregulin), as well as c-erb-B2, c-erb-B3, and c-erb-B4 [10,11,17–19]. These cancers also overexpress basic fibroblast growth factor (FGF-2), acidic FGF (FGF-1), keratinocyte growth factor (KGF), FGF-5, PDGF B chain (but not A chain), insulin-like growth factor-I (IGF-I), the EGF-like growth factor Cripto, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), all 3 mammalian transforming growth factor beta (TGF-β) isoforms, bone morphogenetic protein-2 (BMP-2) and activin βA [14,15,20–29]. Many, but not all of the corresponding receptors are concomitantly overexpressed. For example, there is overexpression of PDGF receptor α and β, the IGF-1 receptor, MET (the receptor that binds HGF), the 2 Ig-like form of type I FGF receptor (FGFR-1), and the type II TGF-β receptor (TβRII) but not the insulin receptor [16,21,23,26,30–33]. IGF-II and insulin are not overexpressed in PDAC [21], whereas the type I TGF-β receptor (TβRI) is under-expressed [31–33]. Thus, there is selective overexpression of specific receptors and their ligands in PDAC, and this concomitant overexpression leads to the creation of aberrant paracrine and autocrine pathways that confers a distinct growth advantage to pancreatic cancer cells.

The clinical importance of the above observations is underscored by numerous observations. For example, the concomitant presence in the cancer cells of EGFR and either EGF or TGF-α is associated with disease progression and decreased survival of PDAC patients [34]. Overexpression of c-erbB3 [19], FGF-2 [20] or TGF-β [35] is associated with decreased patient survival. The aberrant cytoplasmic localization of amphiregulin [36] is also associated with decreased patient survival. Dominant negative inhibition of either EGFR or FGFR-1 markedly attenuates pancreatic cancer cell growth [37–39]. Expression of a cyclin D1 antisense construct in pancreatic cancer cells lowers cyclin D1 levels in these cells, attenuates their growth *in vitro*, and blocks their tumorigenicity in vivo [40]. EGFR blockade with an anti-EGFR antibody attenuates pancreatic tumor growth, and inhibition of EGFR tyrosine kinase activity suppresses pancreatic tumor angiogenesis [41,42]. Together, these findings are among many that support the hypothesis that tyrosine kinase receptors and ligands have an important role in PDAC.

VEGF family of growth factors and their receptors

VEGF-A, also called "vascular permeability factor", is a homodimeric heparin-binding glycoprotein [43–45]. Five major VEGF-A isoforms having 121, 145, 165, 189 and 206 amino acid residues, respectively, arise as a result of alternative splicing from a single gene [46,47]. VEGF-A_{121} and VEGF-A_{145} are usually secreted while VEGF-A_{189} and VEGF-A_{206} are almost completely sequestered in the extracellular matrix [47]. VEGF-A_{165} is half secreted and half bound to the cell surface and the extracellular matrix [48]. All 5 isoforms are mitogenic toward vascular endothelial cells and induce vascular permeabilization. Additional VEGF isoforms and VEGF-related genes have been identified, including VEGF-B [49,50], VEGF-C [51], VEGF-D [52], VEGF-E [53] and placenta growth factor [54]. Direct evidence for the role played by VEGF-A in embryonic vasculogenesis and angiogenesis was also demonstrated in VEGF-A gene knockout studies [55,56], in which loss of a single VEGF-A allele in mice resulted in embryonic lethality between day 11 and 12. Angiogenesis and blood-island formation were impaired, resulting in severe developmental anomalies. This heterozygous lethal phenotype is indicative of the tight dose-dependent regulation of embryonic vessel development by VEGF-A [55,56]. VEGF-A is also required for the cyclical blood vessel proliferation in the female reproductive tract and for longitudinal bone growth and endochondral bone formation in postnatal development [43]. Together, these observations indicate that VEGF-A has an important role in embryogenesis, development, and tissue remodeling.

VEGF-A stimulates endothelial cell proliferation through binding to two related tyrosine kinase receptors, VEGFR-1 (flt-1) VEGFR-2 (flk-1/KDR), on the surface of endothelial cells, with most of the mitogenic effects taken to occur via VEGFR-2 [57–59] (57–59). A third high affinity VEGF receptor, termed VEGFR-3 (Flt4), is expressed in lymphatic vessels [60,61]. It is activated by VEGF-C, which can be processed to a form that also binds to VEGFR-2 [57–61]. Furthermore, placenta growth factor and VEGF-B bind only VEGFR-1, whereas VEGF-D, like VEGF-C, interacts

with both VEGFR-2 and VEGFR-3 [57–61]. However, VEGF-E binds only to VEGFR-2 [59]. All three VEGFRs are class III transmembrane protein tyrosine kinases that possess seven immunoglobulin-like sequences in their extracellular domains and a kinase insert in their intracellular domains [57–61]. In addition, neuropilin-1 (Np-1), a neuronal guidance molecule for axons in the developing nervous system, also acts as a co-receptor for VEGF-A_{165} (but not for VEGF-A_{121}), PlGF-2, VEGF-B and VEGF-E [62]. Np-1 is a non-tyrosine kinase transmembrane protein whose overexpression in transgenic mice is associated with various abnormalities, including excess capillary and blood vessel formation [63]. The closely related neuropilin-2 (Np-2) also binds VEGF-A_{165} (but not VEGF-A_{121}), as well as VEGF-A_{145} and PlGF-2, strongly implying that both Np-1 and Np-2 in angiogenesis [62–64].

Gene knockout studies have shown that both VEGFR-$1^{-/-}$ and VEGFR-$2^{-/-}$ mice die *in utero* between day 8.5 and 9.5 [65,66]. In VEGFR-$1^{-/-}$ mice, endothelial cells developed in both embryonic and extra-embryonic sites but failed to organize into normal vascular channels [65]. In VEGFR-$2^{-/-}$ mice, hematopoietic precursors were severely reduced, yolk-sac blood islands were absent, organized blood vessels failed to develop throughout the embryo or the yolk sac [66]. Furthermore, double knockouts for Np-1 and Np-2 die *in utero* between day 8.5 and 9.5 [67]. They exhibit avascular yolk sacs, and mice that are deficient for Np-1 but heterozygous for Np-2, or deficient for Np-2 but heterozygous for Np-1, die at day 10 to 10.5 and exhibit diffuse vascular abnormalities that are more marked than either Np-1 or Np-2 single knockouts [67]. Together, these observations suggest that VEGFR-1 and VEGFR-2 are essential for embryonic vasculature development, whereas VEGFR-3 is essential for lymphangiogenesis, and that Np-1 and Np-2 are as important as the other components of the VEGF pathway in embryonic angiogenesis.

Angiogenesis in cancer

Tumor angiogenesis is often the consequence of an angiogenic imbalance in which pro-angiogenic factors predominate over anti-angiogenic factors [68–71]. Furthermore, angiogenesis is essential for growth and metastasis of most solid malignancies, and VEGF-A is believed to be critical for tumor angiogenesis [72,73]. Thus, secretion of bioactive VEGF-A by cancer cells may be directly involved in tumor progression [43]. For example, ovarian cancer cells secrete large amounts of bioactive VEGF-A that may play a crucial role in the genesis of ascitic fluid accumulation, angiogenesis and tumor induced immunosuppression in ovarian cancer patients [74]. In high grade gliomas, bioactive VEGF-A secreted by the glioma cells may account for the histopathological and clinical features of these tumors, including such characteristics as marked tumor angiogenesis and increased cerebral edema [75,76].

VEGF-A is expression is induced by multiple mechanisms. These include mutant K-*ras* and mutant *p53*, the von Hippel Lindau gene product, growth factors such as FGF-2 and TGF-β, hypoxia, and transcription factors such as hypoxia inducible factor 1 alpha and SP1 [77–81]. VEGF-A is up-regulated in many tumors including mammary, colorectal, renal, liver, ovarian and gastric carcinomas and gliomas [43], and its overexpression has been correlated with poor prognosis. For example, breast cancer patients with metastatic disease whose tumors exhibit increased angiogenesis have a worse prognosis than the corresponding patients whose tumors do not exhibit increased angiogenesis [82]. Furthermore, suppression of VEGF-A functions inhibits tumor growth in animal models as demonstrated with a dominant negative VEGFR-2, soluble VEGFR-1, neutralizing anti-VEGF-A antibody, VEGF-A anti-sense expression, anti-VEGFR-1 or anti-VEGFR-2 ribozymes, tyrosine kinase inhibitors of VEGFR-2, and anti-VEGFR-2 antibodies [83–92].

Role of VEGF in pancreatic cancer angiogenesis

Although PDAC is not a grossly vascular tumor, this malignancy often exhibits enhanced foci of endothelial cell proliferation. Moreover, several [24,93,94], but not all [95] studies, have reported a positive correlation between blood vessel density, tumor VEGF-A levels, and disease progression in PDAC, raising the possibility that VEGF-A may have an important role in this disease. However, PDACs overexpress multiple additional mitogenic growth factors which are also angiogenic (Table 1), such as EGF, TGF-α, HGF, FGFs such as FGF-1, FGF-2, and FGF-5, and PDGF-beta [6,96]. Therefore, while VEGF-A is of crucial importance in promoting the growth and metastasis of pancreatic cancer cells in PDAC, other factors are most likely also involved in this process. Nonetheless, it has been demonstrated that pancreatic cancer cells secrete biologically active VEGF-A [25], and the cancer cells in PDAC as well as pancreatic cancer cell lines sometimes express VEGFR-1 and/or VEGFR-2 [97]. Moreover, some of these cells may be growth stimulated by VEGF-A in cell culture [97,98], and the major angiogenic agent toward human dermal microvascular endothelial cells (HDMEC) that is produced by T3M4 and PANC-1 human pancreatic cancer cells is VEGF-A, since the mitogenic activity of conditioned medium from these cells can be nearly completely suppressed by neutralizing anti-VEGF-A antibodies [99]. Together, these observations suggest that by promoting angiogenesis VEGF-A enhances tumor spread and metastasis in this malignancy.

In support of the above conclusion, it has been demonstrated that anti-angiogenic therapy is effective at sup-

Table 1: Examples of Angiogenic Growth Factors that Are Overexpressed in Human Pancreatic Cancer and their Cognate Receptors

Growth Factors Activating Tyrosine Kinase Receptors	Receptor
VEGF-A	VEGFR-1 and VEGFR-2
VEGF-C	VEGFR-3
EGF, TGF-α, HB-EGF	EGF receptor
FGF-1, -2, -5	FGF receptors, types 1 and 2
PDGF B chain	PDGF receptors α and β
IGF-1	IGF-1 receptor
Hepatocyte growth factor	MET
Growth Factors that Activate Serine-Threonine Kinase Receptors	
TGF-β1, -2, -3	Type II TGF-β receptor
Pro-Angiogenic Chemokines	
IL-8	CXCR1 and CXCR2
Mip 3α	CCR6

pressing tumor growth in animal models of PDAC. Thus, the anti-angiogenic agent TNP-470 reduces neoangiogenesis in tumors formed by pancreatic cancer cell lines, and decreases tumor growth and metastasis [99]. Suppression of VEGF-A expression with a VEGF-A antisense construct and with a VEGF directed ribozyme markedly attenuates tumorigenicity in nude mice and formation of hepatic metastases [25,100]. VEGF-A fused to diphtheria toxin (DT-VEGF) internalizes in target cells via VEGFRs, inhibits protein synthesis, and suppresses the growth of HUVEC endothelial cells, thereby decreasing the volume and microvessel density in tumors formed by pancreatic cancer cells [101]. Adenoviral vectors carrying sequences encoding soluble VEGFR-1 and VEGFR-2 [102,103], or the VEGFR tyrosine kinase inhibitor PTK 787 [104], also inhibit the growth of growth and/or metastasis of pancreatic cancers in mouse models. These findings underscore the importance of the angiogenic process in PDAC, support the hypothesis that VEGF-A exerts a crucial role in this regard, and raise the possibility that VEGF-A may exert direct effects on pancreatic cancer cells *in vivo*.

VEGF-A can also act as a survival factor for endothelial cells, rendering these cells more radioresistant [105]. It can also promote the survival of leukemic cells, certain tumor cells and hematopoietic stem cells [106–108]. In addition, VEGF-C is also overexpressed in PDAC, and this overexpression has been correlated with enhanced lymph node metastasis [109]. Thus, various members of the VEGF family of ligands may contribute to the growth and metastasis of pancreatic cancer cells through a variety of mechanisms.

Additional mechanisms for promoting pancreatic cancer angiogenesis

Although VEGF appears to be of paramount importance for the angiogenic process in PDAC, these cancers express many other pro-angiogenic factors (Table 1). As in the case of VEGF, some of these growth factors activate tyrosine kinase receptors that are expressed in endothelial cells within the pancreatic tumor mass, such as EGFR [17]. The importance of tyrosine kinase receptors other than VEGFR in pancreatic cancer angiogenesis is underscored by recent observations that inhibition of EGFR tyrosine kinase activity suppresses pancreatic tumor angiogenesis [42], and that NK4, an antagonist that is composed of the N-terminal hairpin and subsequent four-kringle domains of HGF, is a competitive antagonist for HGF that potently inhibits angiogenesis in tumors formed by SUIT-2 pancreatic cancer cells [110].

Other pro-angiogenic factors that are overexpressed in PDAC include certain chemokines such as Mip3α and interleukin-8 (IL-8), which activate G-protein coupled receptors [111–113]. By contrast, TGF-βs activate serine-threonine kinase receptors [114]. The importance of TGF-βs are pro-angiogenic factors in PDAC is underscored by the recent observation that expression of a soluble TβRII in pancreatic cancer cells interferes with TGF-β actions, attenuates tumor growth and metastasis, and suppresses tumor angiogenesis [Rowland-Goldsmith, 2001 #905; Rowland-Goldsmith MA, 2002 #2548].

Often, there is evidence for cross-talk between the various angiogenic factors. For example, TGF-β1 and plasminogen activator inhibitor-1 (PAI-1) are overexpressed in PDAC [117,118], TGF-β1 induces PAI-1 expression in pancreatic cancer cells [119], and both TGF-β1 and PAI-1 and can promote angiogenesis *in vivo* [120–122]. TGF-βs are initially released as latent molecules that form complexes with latent binding protein (LTBP), and their biological effectiveness is dependent on their activation by such proteins as plasmin, uPA and its receptor, the insulin-like growth factor II (IGF-2) receptor, and tissue transglutami-

nase [123,124]. The IGF-2 receptor, as well as uPA and its receptor are overexpressed in PDAC [125,126], and pancreatic cancer cell lines express tissue transglutaminase [127]. Furthermore, uPA and its receptor, as well as tissue transglutaminase, have been implicated in the angiogenic process [128,129], and the angiogenic potential of TGF-βs may be enhanced by the presence of Smad4 mutations [130], which are frequent in PDAC. uPA can transctivate EGFR [131], and EGFR activation can induce the expression of VEGF and the pro-angiogenic chemokine interleukin-8 [132,133]. Taken together, these observations suggest that multiple pathways interact to enhance angiogenesis in PDAC.

The pancreatic microenvironment may also serve to promote tumor angiogenesis [134]. In addition, as a consequence of the existence of a continuous intra-pancreatic portal circulation, pancreatic cancer cells may be exposed to high levels of islet cell derived hormones such as insulin and growth factors such as TGF-βs [135]. High insulin levels bind and activate the IGF-1 receptor, which can then promote angiogenesis [136,137]. Furthermore, islet cell derived TGF-βs may enhance matrix metalloprotease-9 (MMP-9) and VEGF expression in PDAC [31,138], and suppress PTEN expression [139]. MMP-9 enhances tumor angiogenesis [140] whereas PTEN, a phosphatase with specificity for 3-phosphorylated inositol phospholipids, has been implicated in the suppression of tumor angiogenesis [141].

Conclusion

PDAC is a biologically aggressive malignancy that has a propensity to spread locally and metastasize distally. While not grossly vascular, these cancers exhibit foci of micro-angiogenesis and overexpress multiple pro-angiogenic factors. VEGF and related ligands represent a crucial component of this pro-angiogenic switch, as evidenced by the presence of high levels of VEGF in ascitic fluid of PDAC patients [142], the correlation between high serum VEGF levels and disease recurrence post-operatively [143], and the observation that high VEGFR-2 levels are associated with a worse prognosis in this disease [144]. Therefore, mechanisms that target VEGF and the various pathways that enhance the angiogenic process in PDAC [145] may ultimately be of great therapeutic benefit in patients with unresectable disease as well as following surgery to prevent disease recurrence.

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