3. Pancreatic stellate cells and fibrosis

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Abstract. Pancreatic cancer is characterised by a prominent desmoplastic/stromal reaction. It is now known that pancreatic stellate cells (PSCs) are the principal source of fibrosis in the stroma and interact closely with cancer cells to create a tumor facilitatory environment that stimulates local tumor growth and distant metastasis. Pancreatic fibrosis is initiated when PSCs become activated and undergo morphological and, more importantly, functional changes so that the rate of extracellular matrix (ECM) deposition exceeds the rate of ECM degradation in the gland. It is now well established that pancreatic cancer cells activate PSCs leading to increased fibrosis. This chapter summarises recent advances in our understanding of the role of fibrosis in pancreatic cancer, with particular reference to the central role played by pancreatic stellate cells. An improved knowledge of PSC biology has the potential to provide an insight into pathways that may be therapeutically targeted to inhibit PSC activation, thereby inhibiting the development of fibrosis in pancreatic cancer and interrupting stellate cell - cancer cell interactions so as to retard cancer progression.

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Introduction

Pancreatic cancer is a devastating disease with a dismal prognosis. It is the fourth leading cause of cancer related death in Western societies, with a 5 year survival rate of less than 5% [1-2]. A major reason for the poor clinical outcome is its well known resistance to chemotherapeutic agents [3-5]. Despite aggressive treatment regimes, little improvement in patient survival has occurred in the last decade. In fact, the best chemotherapeutic treatments currently only prolong life by ~6-12 weeks [6]. Therefore, alternative approaches are urgently needed to improve the outcome of this condition. Until recently, studies of the pathogenesis of pancreatic cancer have mainly focused on the molecular biology of the tumor cells themselves. However, there is now significant evidence showing that the intense stromal/desmoplastic reaction around tumor elements (a feature of the majority of pancreatic cancers) plays an important role in tumor progression [7-12]. Our group established that the cells responsible for producing the stromal reaction in pancreatic cancer are pancreatic stellate cells (PSCs, the key fibrogenic cells in the pancreas) [13]. Our group and others have identified (using in vitro and in vivo approaches) significant bidirectional interactions between PSCs and cancer cells which facilitate local tumor growth and distant metastasis [13-18].

The microenvironment of pancreatic cancer

A key histopathological feature of pancreatic cancer which is associated with its innate clinical and biological aggressiveness is its pronounced desmoplastic (stromal) reaction which is now considered an alternative therapeutic target in pancreatic cancer. Stroma production is stimulated by cancer-cell derived growth factors including transforming growth factor-β (TGFβ), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) [11]. The desmoplastic reaction is composed of extracellular matrix (ECM) proteins, primarily type I and III collagen, fibronectin and proteoglycans; small endothelium lined vessels; and a diverse population of cells including inflammatory cells, fibroblasts and stellate cells [19]. The stroma can form up to 90% of the tumor volume, a property which is unique to pancreatic cancer [12, 20]. Pancreatic cancer cells in vitro show a similar response to chemotherapeutic agents as cell lines derived from other solid tumors [20]. However, pancreatic cancer patients have a limited response to drugs such as paclitaxel compared to breast and prostate cancer patients, suggesting that the
unique tumor microenvironment in pancreatic cancer plays a role in chemoresistance [20].

Whilst stromal cells do not exhibit the genetic transformations seen in malignant pancreatic cancer cells, they are altered by cytokines and growth factors secreted by inflammatory cells and tumor cells [21]. Reciprocally, the stromal cells promote tumor cell migration, growth, invasion and resistance to drugs and apoptosis [21]. As mentioned above, PSCs have recently emerged as the principal regulator of the desmoplastic reaction in pancreatic cancer [13], as well as key effector cells in pancreatic fibrosis during necroinflammation in chronic pancreatitis [22-24] a known risk factor for pancreatic cancer [25]. Erkan et al [26] observed through staining pancreatic cancer tissue sections of patients for alpha smooth muscle actin (α-SMA the cytoskeletal protein marker for PSC activation) and collagen that a high activated stroma index (α-SMA/collagen) correlated with a poor prognosis. Furthermore, the extensive ECM deposition by PSCs in pancreatic cancer causes distortion and compression of tumor vasculature by fibrous tissue which contributes to tumor hypoxia, a determinant of chemoresistance [27-30].

The biology of pancreatic stellate cells

First isolated in 1998 [31-32], PSCs are resident cells of the pancreas predominantly located in the periacinar, periductal and perivascular spaces of the pancreas [31-33]. A recent study by Erkan et al [34], demonstrated that PSCs have a similar transcriptional fingerprint to hepatic stellate cells (HSCs, counterpart cells in the liver, key mediators of liver fibrosis) and therefore may have a similar origin. This study identified that collagen type 11α1 (COL11α1) may be a novel PSC specific marker with up to 65-fold higher expression levels in PSCs compared to HSCs [34]. Similar results were obtained by Buchholz et al [35] showing that PSCs only differed from HSCs with respect to at least 29 genes. Compared to HSC, PSCs exhibit increased periostin, serine protease 11, integrin-α7, connective tissue growth factor, cytoskeletal elements (actinγ2, desmoplakin) and hypoxia inducible factor 1α subunit. Despite these similarities, no consensus in the field has been reached as to the exact origin of PSCs, with both mesenchymal and neuroectodermal origin suggested.

In health, PSCs comprise ~4–7% of pancreatic cells and exist in their quiescent phenotype where they contain numerous cytoplasmic vitamin A containing lipid droplets, and express specific markers such as desmin and glial fibrillary acidic protein (GFAP) [13, 23]. In a normal pancreas, PSCs
may play a role in normal tissue architecture by regulating extracellular matrix turnover, given their known synthesis of ECM proteins and matrix degrading enzymes (matrix metalloproteinases [MMPs] and tissue inhibitors of metalloproteinases [TIMPs]) [36]. Recent novel evidence from our group also demonstrated that PSCs can synthesize and secrete the neurotransmitter acetylcholine, which may play a role in mediating enzyme secretion from acinar cells [37]. This feature also supports a potential neuroectodermal origin.

In response to pancreatic injury, PSCs transform into an active myofibroblast-like phenotype, under the influence of activating factors such as alcohol and oxidant stress [7, 22, 38] or from products of injured cells including pro-inflammatory cytokines and growth factors (Figure 1) [39-43]. This transdifferentiation is accompanied by a loss of cytoplasmic vitamin-A lipid droplets and increased cytoskeletal protein α-SMA expression [31]. Activated PSCs subsequently develop functional alterations including: 1) increased proliferation and migration [33, 39, 42, 44-45]; 2) synthesis of excessive ECM proteins (collagen, fibronectin, laminin) as well as matrix metalloproteinases and their inhibitors [13-14, 36, 39, 45]; and 3) secretion of growth factors and cytokines which exert both paracrine and autocrine effects that enhance cell growth and migration (Figure 1) [33, 46-50].

Transformation of PSCs from a quiescent to an activated phenotype has been the subject of intense study in recent years. Several signalling pathways/molecules that mediate this process have been identified [51]. These include mitogen activated protein kinases (MAPK), phosphatidylinositol kinase (PI3K), protein kinase C (PKC), peroxisome proliferator activated receptor gamma (PPARγ), the JAK-STAT pathway and the transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1).

**Role of pancreatic stellate cells in pancreatic fibrosis**

Although this chapter focuses on the production of fibrous ECM by PSCs (the major cells mediating fibrosis in the pancreas), it is to be noted that other potential sources of fibrosis are present in the pancreas. The fibrotic matrix in pancreatic cancer was initially thought to be produced as a result of chronic injury as a host barrier against tumor invasion. However, evidence now indicates that the fibrotic reaction in pancreatic cancer is essential for tumor promotion and progression. The fibrotic ECM provides a physical scaffold and is a sink for soluble growth factors capable of influencing pancreatic cancer cell growth, survival and motility. Fibrogenesis is a dynamic process which is potentially reversible, at least in its early stages. A significant improvement in
Pancreatic stellate cells and fibrosis

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Figure 1. Pancreatic stellate cell activation. In health, PSCs are in a quiescent state, where they contain numerous cytoplasmic vitamin A containing lipid droplets. In pancreatic cancer or chronic pancreatitis PSCs transform into an activated myofibroblast-like cell, which is accompanied by a loss of vitamin A lipid droplets. PSCs are activated by growth factors and cytokines secreted by injured acinar cells and ductal cells, pancreatic cancer cells, endothelial cells and inflammatory cells. Similarly, ethanol and its metabolite acetaldehyde as well as oxidant stress activate PSCs. PSC activation leads to production of fibrous extracellular matrix (ECM) proteins (collagen, laminin and fibronectin), increased migration to areas of injury and increased proliferation. Autocrine factors are also secreted by PSCs which perpetuate their activation. TGF = transforming growth factor; PDGF = platelet derived growth factor; VEGF = vascular endothelial growth factor; TNF = tumor necrosis factor; IL = interleukin; EMMPRIN = extracellular matrix metalloproteinase inducer; ET-1 = endothelin-1; CTGF = connective tissue growth factor; COX-2 = Cyclooxygenase.

in our understanding of the molecular mechanisms of pancreatic fibrosis were largely made possible by the identification, isolation and characterisation of PSCs [31-32].

A number of growth factors and proinflammatory cytokines including TGFβ1, platelet-derived growth factor (PDGF), tumor necrosis factor α (TNFα) and the interleukins 1 and 6 are known to be upregulated during
necroinflammation of the pancreas. Therefore, the concept that PSCs are activated by proinflammatory cytokines released during pancreatic injury has been tested by several investigators. It is well established that upon exposure to PDGF, cultured PSCs exhibit increased cell proliferation and migration [39, 41, 43, 45, 52-53]. The profibrogenic cytokine TGFβ has been shown to increase the expression of α-SMA and the ECM proteins collagen and fibronectin in PSCs [39, 41, 45, 50]. The proinflammatory cytokines TNFα, IL1 and IL6 have also been shown to activate PSCs [42]. PSCs are also activated by reactive oxygen species (generated within the pancreas by oxidative metabolism of alcohol and/or necroinflammation). Furthermore, PSCs can secrete TGFβ1 [50], PDGF [11], connective tissue growth factor (CTGF) and endothelin-1 [54]. These autocrine signals result in perpetuation of stellate cell activation and increased production of fibrous ECM.

Dual staining (using Sirius red for collagen and immunostaining for αSMA) of sections from both human and rat pancreas has shown that there is co-localisation of αSMA positive staining with bands of fibrosis (collagen) suggesting the presence of activated PSCs in fibrotic areas [24]. Increased numbers of PSCs in areas of fibrosis have been reported using the TNBS model of pancreatic fibrosis. Haber et al [24] have demonstrated increased staining for the stellate cell marker desmin in areas of fibrosis indicating that PSC numbers are increased in areas of injury. This increase in PSC number may be due to local proliferation and or migration of PSCs to affected areas. Studies using immunostaining for αSMA and in situ hybridization for collagen messenger RNA have also indicated that activated PSCs are the primary source of collagen in the fibrotic pancreas [39]. More recently, similar experimental approaches have indicated that activated PSCs are the principal source of collagen in the stromal reaction around pancreatic cancers [13, 55]. Thus evidence from in vivo studies (using human tissue and animal models) as well as in vitro studies (using cultured PSCs) supports the concept that PSCs are key players in the fibrogenesis process in the pancreas.

The role of cancer-stellate cell interactions in tumor progression

As mentioned above, there is now a significant body of evidence showing that a bidirectional interaction exists between PSCs and pancreatic cancer cells which favours tumor progression (Figure 2) [13-18]. In summary, via production of mitogenic and fibrogenic mediators, pancreatic cancer cells attract and promote the activation, proliferation and motility of PSCs [13-14, 17-18, 56]. Pancreatic cancer cells also regulate the capacity of PSCs to
Figure 2. A schematic representation of the bi-directional interaction between pancreatic cancer cells and pancreatic stellate cells (PSCs). Pancreatic cancer cells secrete several factors which participate in the recruitment and activation of PSCs. When activated by cancer cells, PSCs have increased proliferation, ECM deposition (predominantly fibrous collagen) and migration. In turn, PSCs secrete factors which enhance the survival of pancreatic cancer cells (via increased proliferation and reduced apoptosis). PSCs secrete MMPs which increase the invasion of pancreatic cancer cells. While the fibrotic microenvironment produced by PSCs increases epithelial-mesenchymal transition (EMT) and chemoresistance of pancreatic cancer cells. This bi-directional interaction between PSCs and cancer cells favours tumor progression and metastasis. TGF = transforming growth factor; PDGF = platelet-derived growth factor; VEGF = vascular endothelial growth factor; FGF = fibroblast growth factor; TNF = tumor necrosis factor; EMMPRIN = extracellular matrix metalloproteinase inducer; SDF = stromal derived factor; IGF = insulin growth factor; ECM = extracellular matrix; COX = cyclooxygenase; EGF = epidermal growth factor.

remodel the ECM [14]. Bachem et al [14] demonstrated that supernatant derived from human pancreatic cancer cell lines increased PSC proliferation and matrix synthesis, in a dose dependent manner. These two effects were suppressed when neutralising antibodies against the growth factors PDGF, FGF2, and TGFβ1 were introduced, confirming the influence of mediators released by pancreatic cancer cells on PSC activity [14]. Cancer cells also have the capacity to secrete the ECM metalloproteinase inducer (EMMPRIN), which results in increased MMP2 secretion by PSCs [57].
TGFβ1 has also been shown to stimulate MMP2 secretion by PSCs [36]. MMP2 has been associated with the invasive phenotype of pancreatic cancer cells and is essential for degradation of normal basement membrane for cancer progression.

Bachem et al [14] were the first to provide evidence of an interaction in vivo and reported that subcutaneous coinjection of PSCs and cancer cells into the flanks of nude mice resulted in larger tumors with a significant stromal reaction than those produced by injection of cancer cells alone. In another study by the same group, co-injection of PSCs with cancer cells overexpressing the serine protease inhibitor SERPINE2 into nude mice was reported to result in increased tumor growth [58]. These findings suggested that increased ECM (in particular, fibrillar collagen) deposition as a consequence of protease inhibition might facilitate cancer progression, a concept supported by the observation that ECM proteins protect pancreatic cancer cells from apoptosis. Although these studies provided important evidence of stromal-tumor interactions, subcutaneous models are not an ideal choice for studying pancreatic cancer because they do not allow the assessment of tumor behaviour within the appropriate microenvironment. More recently, our laboratory developed an orthotopic model of pancreatic cancer which overcomes some of the limitations of subcutaneous models. In vivo, mice receiving a co-injection of pancreatic cancer cells and human PSCs (hPSCs) into the pancreas, histologically exhibited dense bands of fibrosis which correlated with the presence of activated PSCs as assessed by α-SMA expression [17].

Simultaneously, pancreatic cancer co-opts PSCs to form a growth permissive and tumor facilitatory environment. By producing and sequestering multiple growth factors, cytokines and ECM components, PSCs actively participate in pancreatic cancer proliferation, migration, invasion, metastatic dissemination and survival [16-18]. Watanabe et al [59] reported that more extensive intratumoral fibroblastic cell proliferation correlates with a poorer disease outcome in pancreatic cancer patients. Using an in vivo orthotopic mouse model of pancreatic cancer, our laboratory demonstrated that intra-pancreatic co-injection of human PSCs with pancreatic cancer cells yielded larger tumors and more extensive local and distant metastases compared with injections of tumor cells alone [17]. Furthermore, PSCs co-migrated with pancreatic cancer cells to metastatic sites where they are postulated to play a role in seeding and supporting cancer growth [18]. Similarly Hwang et al [16] showed that the incidence of tumor formation, distant metastases and mean pancreatic weight were significantly augmented with increasing proportion of human PSC to pancreatic cancer cells. In line with these observations in vivo, human PSC conditioned medium stimulated
pancreatic tumor cell proliferation and colony formation in vitro [16]. Direct and indirect co-culture systems have further demonstrated increased migration, invasiveness and proliferation of pancreatic cancer cells in the presence of human PSCs [17, 60]. These findings support the notion that PSCs play a pivotal role in tumor progression.

**The hypoxia fibrosis cycle**

An implication of the extensive desmoplastic reaction in pancreatic cancer is intratumoral hypoxia, a major determinant of pancreatic cancer chemoresistance [15, 29, 61]. Hypoxia arises when the proliferating tumor outgrows its ambient vascular supply. Although PSCs harbor proangiogenic abilities via the secretion of vascular endothelial growth factor (VEGF), FGF, periostin and collagen type I, it is predominantly fibrogenic, and the extensive periacinar deposition of ECM proteins has been shown to distort the normal parenchymal architecture and compress the fine capillary network therefore limiting oxygen diffusion [15, 30]. Concomitantly, PSCs amplify the production of endostatin by pancreatic cancer cells (an endogenous inhibitor of angiogenesis), which overwhelms the angiogenic capacity of both PSCs and pancreatic cancer and attenuates microvessel density [15]. Essentially, the vasculature in pancreatic cancer becomes highly disorganised, dysfunctional and permeable [62-63]. Moreover, as hypoxia has been shown to perpetuate PSC proliferation and activation in vitro and in vivo, intra-stromal hypoxia sustains the periacinar deposition of ECM [15, 64]. Consequently, at the invasive front of the reactive stroma, cytokines and growth factors secreted by pancreatic cancer cells, and fibrosis which leads to tumor hypoxia, perpetuates PSC activity and propagates fibrotic changes beyond the actual tumor itself (i.e. inducing a hypoxia-fibrosis cycle, Figure 3) [19].

In pancreatic cancer, the abnormal blood flow dynamics of the tumor vasculature and the dense stroma imposes a considerable diffusion barrier to systemic drug delivery (Figure 3). Using a transgenic mouse model of pancreatic cancer which is refractory to gemcitabine, Olive et al [30] demonstrated that pancreatic tumors had a dysfunctional vasculature and that delivery of autofluorescent drugs such as doxorubicin into the pancreatic cancer tissue was impaired compared with control tissues. Depletion of the pancreatic cancer stroma by inhibiting the paracrine hedgehog signalling by pancreatic cancer cells to PSCs, however, potentiated intratumoral vascular density, augmented the delivery of gemcitabine and transiently enhanced pancreatic cancer chemosensitivity [30, 65]. Recent clinical evidence by Komar et al [66] in human patients quantified blood flow and metabolic activity of pancreatic tumors using oxygen-15 labelled water [\(^{15}\)O]-H\(_2\)O and
Figure 3. Pancreatic stellate cell mediated hypoxia fibrosis cycle in the chemoresistance of pancreatic cancer. PSC activation by pancreatic cancer cells leads to increased fibrosis production, which results in decreased microvessel density and abnormal vasculature. This altered vasculature leads to intrastromal and intratumoral hypoxia. In response to hypoxia and cancer cell secretions, PSCs proliferate and sustain ECM deposition, inducing a hypoxia-fibrosis cycle. Hypoxia also increases PSC activation and epithelial-mesenchymal transition (EMT), both of which contribute to overall chemoresistance. In addition, the dense fibrosis and dysfunctional vasculature inhibit the delivery of chemotherapy agents into the tumor.

[18F]-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT imaging. This study demonstrated reduced blood flow and high metabolic activity in pancreatic tumors compared to normal pancreatic tissue. In addition, a high ratio of glucose uptake to blood flow was a predictor of poor prognosis [66]. The authors suggested that a high metabolic activity combined with low blood flow is indicative of a hypoxic tumor microenvironment [66]. These findings strongly suggest that in pancreatic cancer, the fibrotic and highly avascular microenvironment synergistically: 1) reduce drug delivery via the poorly perfused blood into the tumor; 2) causes the sequestering of drugs in the peritumoral stroma; and 3) leads to a decline in the effective intracellular drug concentration within pancreatic cancer cells, compromising therapeutic success.

Concomitantly in this hypoxic environmental niche, there is a reduction in the delivery of nutrients and the clearance of by products from anaerobic metabolism thus inhibiting pancreatic cancer cell proliferation and limiting
the effectiveness of cytotoxic drugs which are incorporated during the S-phase of the cell cycle [20]. Furthermore, hypoxic stress stabilise the hypoxia regulated transcription factor, hypoxia inducible factor-1α (HIF-1α) and upregulate genes associated with angiogenesis, glycolysis, enhanced survival and epithelial-mesenchymal transition, all of which have been shown to contribute to increased chemoresistance [67-70].

In addition to hypoxic mechanisms of chemoresistance, PSC-pancreatic cancer cell interactions impair responses to chemotherapy. Our laboratory has demonstrated that human PSC secretions are capable of conferring a chemoresistant phenotype by suppressing H$_2$O$_2$-induced apoptosis and increasing survival of pancreatic cancer cells [17]. Muerkoster et al [71] demonstrated that pancreatic cancer cells became less sensitive toward treatment with etoposide when co-cultured with PSCs. These results were supported by Hwang et al [16] who demonstrated that gemcitabine and radiation therapy were less effective in pancreatic cancer cells treated with conditioned medium of human PSCs. Interestingly the presence of ECM proteins produced by PSCs promoted resistance of pancreatic cancer cells to 5-fluorouracil (5-FU), cisplatin and doxorubicin [72]. These results suggest that tumor-stromal interactions may promote the survival of tumor cells in the presence of chemotherapy. However little is known about the proteins mediating PSC-induced chemoresistance of pancreatic cancer cells.

**Role of pancreatic stellate cells and fibrosis in epithelial-mesenchymal transition**

Epithelial-mesenchymal transition (EMT) is associated with metastatic spread and resistance to apoptosis. A characteristic feature of EMT is the switch of the epithelial-specific junction protein E-cadherin to mesenchymal N-cadherin. A recent study by Kikuta et al [73] showed that PSCs promoted EMT (decreased E-cadherin and increased vimentin and Snai-1) in pancreatic cancer cells using a co-culture assay. Although the exact mechanisms and in vivo evidence is lacking in this area, it is likely that the altered vascularity and increased fibrosis resulting from PSC activation leads to hypoxia, which is known to increase EMT in cancer cells (Figure 3).

**Targeting pancreatic stellate cell mediated fibrosis: A potential therapy**

PSCs produce the stromal reaction in pancreatic cancer, provide a growth-permissive microenvironment for cancer cells and facilitate distant
spread, as outlined above. PSCs are activated (characterised by loss of vitamin A droplets in their cytoplasm and transformation into myofibroblasts) and found in increased numbers in the stromal/fibrotic areas of pancreatic cancer. This increase in PSC numbers may be due to increased activation, local proliferation and/or decreased apoptosis. Potential ways to deplete the stromal reaction are i) inhibit PSC activation or proliferation; ii) induce PSC apoptosis; and/or iii) inhibit fibrous tissue production by PSCs. Below evidence is provided on potential therapeutic targets for depletion of PSC mediated fibrosis, which may as a consequence, reduce cancer progression and increase drug delivery into the tumor.

**Sonic hedgehog (SHH)** has been shown to promote desmoplasia [65] and is over expressed in cancer cells of human pancreatic tumors [74]. Tumor cells secrete SHH, which in turn stimulates stromal cells. Bailey et al [65] demonstrated that injection of pancreatic cancer cells over expressing SHH into the pancreas of mice resulted in significantly increased desmoplasia. This was attributable to increased SMA positive fibroblasts (PSCs) and the effect was blocked using a SHH antibody [65]. A recent ground breaking article, showed that pharmacological targeting of the desmoplastic stroma by inhibition of sonic hedgehog signalling, facilitated increased drug delivery into the pancreatic tumors of transgenic mice and increased survival [30]. It is highly likely that this therapeutic approach targeted PSCs. Evidence for this comes from the fact that Bailey et al [75] demonstrated that SMA-positive myofibroblasts (or PSCs) expressed Gli1 (hedgehog transcription factor) in tumours over expressing SHH, indicating that these cells are responsive to stimulation with SHH. Further support was recently provided by Walter et al [76] who demonstrated that the hedgehog receptor Smoothened was upregulated in human pancreatic cancer-associated fibroblasts compared to control fibroblasts and that the cancer associated fibroblasts responded to incubation with recombinant SHH by increased Gli1 expression.

**Periostin** is a matrix-specific protein which is secreted by PSCs and perpetuates their fibrogenic activity [28]. Periostin is induced by hypoxia and is a regulator of angiogenesis. This study showed that periostin increased collagen-I, fibronectin and TGFβ secreted by PSCs. Periostin expression in PSCs was induced by co-culture with pancreatic cancer cells. In cancer cells, periostin stimulated growth and conferred resistance to hypoxia. Therefore, periostin is a potential target to decrease fibrosis which contributes to chemoresistance in pancreatic cancer.

**Halofuginone** is a plant derived alkaloid and is an anti-fibrotic agent. In a thioacetamide-induced liver fibrosis rat model, administration of halofuginone orally before fibrosis induction, prevented the activation of most hepatic stellate cells and remaining cells expressed low levels of
collagen-1 [77]. More importantly, when halofuginone was given to rats with established fibrosis, complete resolution of fibrosis was observed [77]. In a mouse model of cerulein-induced pancreatitis, halofuginone prevented collagen synthesis via inhibiting Smad 3 phosphorylation [78]. In culture, halofuginone inhibited PSC proliferation [78]. Spector et al [79] recently demonstrated that inhibition of PSC activation by halofuginone before implantation with pancreatic cancer cells resulted in reductions in tumor growth and decreased collagen production (subcutaneous and orthotopic). However, the authors did not examine if depletion of the fibrosis by halofuginone increases the delivery of chemotherapeutic agents.

**Endothelin-1** (ET-1) and its receptors are expressed on both PSCs and pancreatic cancer cells [80]. Treatment of PSCs *in vitro* with Bosentan (ET-1 receptor antagonist) significantly decreased collagen synthesis in a dose dependent manner and inhibited PDGF-induced proliferation [80]. In addition, the same study showed that bosentan inhibited cancer cell-induced PSC proliferation (co-culture experiments) as well as PSC-induced cancer cell proliferation [80]. These results warrant further validation using pre-clinical models of pancreatic cancer, especially given the fact that ET-1 receptor antagonist interfered with the bidirectional interaction of PSCs and cancer cells.

Several studies have examined the effect of **anti-oxidants** on PSC activation. Vitamin E prevents PSC activation *in vitro* [22]. While *in vivo* studies involving experimental models of pancreatic fibrosis have reported that anti-oxidants, herbal products with antioxidant properties can significantly reduce pancreatic fibrosis. **γ-Tocotrienol**, a novel, unsaturated form of vitamin E inhibited *in vitro* proliferation of pancreatic cancer cells and potentiated gemcitabine-induced apoptosis [81]. These effects were mediated by suppression of cyclin D1, c-myc, cyclooxygenase-2 (COX-2), Bcl-2, VEGF and chemokine receptor type 4 (CXCR4). γ-Tocotrienol also inhibited tumor growth and enhanced gemcitabine activity in an orthotopic model of pancreatic cancer. In addition, tocotrienols induced activated PSC apoptosis, without affecting quiescent PSCs [82]. Therefore, tocotrienols may be an interesting therapeutic option because they may deplete both the stromal PSCs and pancreatic cancer cells. The use of tocotrienols needs to be further tested in a relevant pre-clinical animal model which has an extensive stromal reaction.

Our laboratory has also demonstrated that exogenous **vitamin A** and its metabolites induced PSC quiescence *in vitro* [83]. Vitamin A and its metabolites are also inhibitors of pancreatic cancer cell growth [84]. However, the vitamin A derivative 13-cis-retinoic acid has been tested in several phase II clinical trial in pancreatic cancer with varying results [85-87].
Cyclooxygenase-2 (COX-2) is an enzyme important in inflammation and the increased production of prostaglandins associated with tumorigenesis [88]. COX-2 is expressed in chronic pancreatitis and pancreatic cancer patients. Celecoxib (a selective COX-2 inhibitor) inhibited COX-2 activity, decreased TGFβ expression, induced metalloproteinase-2 activity and, consequently, prevented and reversed collagen accumulation in a model of liver fibrosis induced by carbon-tetrachloride administration [89]. Activated PSCs express COX-2 when stimulated with TGFβ [90] and co-cultured with pancreatic cancer cells [91]. Pharmacological inhibition of COX-2 in activated PSCs decreased the expression of COX-2, αSMA and collagen I, suggesting that COX-2 might be a relevant target in pancreatic cancer [90]. In addition, COX-2 inhibition also caused increased apoptosis in pancreatic cancer cells [92]. Further support that COX-2 is important in the PSC-cancer cell interaction was provided by Sato et al [93] who demonstrated that COX-2 expression is increased in both PSCs and cancer cells in response to co-culture. This study also showed that blockage of COX-2 (NS-398) partially inhibited PSC-induced invasion of pancreatic cancer cells [93]. Celecoxib has recently been tested in combination with gemcitabine and irinotecan in a phase II clinical trial in patients with advanced pancreatic cancer [94].

Galactin-3 is a member of the β-galactoside-binding protein family, which has been implicated in inflammation and cancer. Silencing the galectin-3 gene in vivo using siRNA inhibited myofibroblast activation and attenuated liver fibrosis in a carbon-tetrachloride liver injury model in mice [95]. A recent study by Jiang et al [96] demonstrated that pancreatic cancer cells express and secrete galectin-3. This study also demonstrated that pancreatic cancer cells stimulated PSC proliferation via galectin-3 (using an antibody for Galectin-3) [96]. The exact mechanism of action of galectin-3 is unknown and inhibitors of galectin-3 have not yet been tested in pre-clinical models of pancreatic cancer.

PDGF is a potent mitogen and chemoattractant for PSCs and therefore may be a potential therapeutic target for fibrosis in pancreatic cancer. PDGF and its receptor PDGFRβ is also upregulated in tumor cells. In fact, the PDGF-antagonist trapidil suppressed PDGF-induced ERK activation and decreased PSC proliferation in vitro [97]. While Masamune et al [98] showed that curcumin (polyphenol compound found in turmeric) resulted in decreased PDGF-induced proliferation of PSCs in vitro and also decreased αSMA expression and collagen-I production. In another study by the same group PDGF-induced PSC proliferation, migration and collagen production were inhibited by epigallocatechin-3-gallate (a polyphenol from green tea)
[99]. Rats with chronic liver injury (bile duct ligation model) were administered hepatic stellate cell (HSC)-specific PDGFR-β shRNA (linked to a GFAP promoter to reduce non-HSC mediated effects) which resulted in decreased liver injury and decreased hepatic fibrosis [100]. This example using the GFAP promoter to target stellate cells is a new powerful tool for cell-specific gene therapy. Furthermore, several PDGF receptor–related antagonists are being developed as potential anti-tumor and anti-stromal agents and have demonstrated promising antitumor activity in both preclinical and clinical settings including imatinib mesylate (Gleevec/ST571), sunitinib malate (Sutent/SU11248), and CP-673,451.

Targeting the pro-fibrogenic TGFβ signalling pathway have showed promise in reducing experimental pancreatic fibrosis in rodents [101-102]. There are several drugs available which target TGFβ synthesis, ligand/receptor binding or receptor kinase signalling. Preclinical studies using TGFβ inhibitors have demonstrated efficacy in reducing metastasis and have shown improvements in cytotoxic drug delivery. Activin A, a member of the TGFβ family activates PSCs and promotes collagen secretion in an autocrine manner [49]. The authors also showed that activin A stimulates TGF-β secretion by PSCs and vice versa TGFβ stimulates activin A secretion by PSCs. Furthermore, follistatin (an endogenous activin A binding protein known to block the effect of activin A) [103] inhibited TGF-β secretion by PSCs and attenuated PSC activation and collagen secretion [49]. These data imply that activin A participates in pancreatic fibrosis and follistatin may be used as an anti-fibrotic agent [49]. Follistatin has not been tested in preclinical models of pancreatitis or pancreatic cancer.

A recent study showed that quiescent PSCs endogenously express albumin (localised in the cytoplasm with lipid droplets) [104]. Upon activation in culture and loss of vitamin A, albumin levels decreased. TGFβ treatment of PSCs also decreased albumin and stably transfected PSCs with albumin are resistant to TGFβ mediated activation [104]. On this point, albumin-bound paclitaxel (nab-paclitaxel;Abraxane) has been shown to bind to albumin receptors in tumor blood vessels and is released into the tumor microenvironment [105]. The albumin nanoparticles were used to deliver paclitaxel to avoid the previously toxic castor oil derivative Cremophor. This drug is reported to result in "stromal collapse" [106] and increased drug delivery into tumors. Although scientists and clinicians are eagerly awaiting clinical trials with abraxane in pancreatic cancer, it may be interesting to examine the effect of albumin-bound paclitaxel on PSC activity in the stroma, especially given the results by Kim et al [104] described above. Albumin itself may be a therapeutic option for reversion of PSCs back to
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their quiescent phenotype and reduce production of pancreatic fibrosis. In addition, novel nanotechnologies may provide us with a way to target a specific cell type in the pancreatic tumor microenvironment (e.g. PSCs) and enhance the effectiveness of other anti-cancer therapies.

Summary Points

- An extensive stromal/fibrous reaction and hypoxic microenvironment are characteristic features of pancreatic cancer.
- Pancreatic stellate cells are key mediators of the fibrosis observed in the desmoplastic reaction of pancreatic cancer.
- A bi-directional interaction exists between pancreatic stellate cells and pancreatic cancer cells which facilitates tumor progression and metastasis.
- Therapeutic intervention in the last decade has had limited impact on improving patient survival in pancreatic cancer. Therefore, it is clear that targeting cancer cells alone is not enough. An alternative approach is to dual target the stromal PSCs and the tumor cells.
- PSCs contribute significantly to the highly chemoresistant nature of pancreatic cancer by producing the extensive fibrous ECM which results in i) altered vasculature and decreased drug delivery to the tumor; ii) decreased sensitivity to chemotherapeutic agents; and iii) increased epithelial-mesenchymal transition.
- Approaches to improve the efficacy of anti-cancer drugs include: i) restoration of vasculature to normal by decreasing the fibrosis; ii) the use of appropriate pre-clinical animal models which mimic human disease (i.e. poorly perfused and hypoxic microenvironment with extensive fibrotic reaction); and iii) targeting the cancer-stellate cell interactions.

Conclusion

Recent developments in our understanding of the prominent stromal reaction in pancreatic cancer has highlighted the importance of the key fibrotic mediators, pancreatic stellate cells. Pancreatic cancer cells recruit PSCs to enhance their growth, modulate the ECM (to increase invasion and promote a chemoresistance phenotype) and aid in metastatic spread. Despite numerous clinical trials (conventional and targeted therapy) in pancreatic cancer over the past decade, only modest improvements in patient survival have been observed. Reasons for this include a lack of understanding chemoresistance mechanisms and inadequate pre-clinical animal models for testing potential therapies. In order to reliably predict outcomes in subsequent human
trials future research should consider the influence of the tumor microenvironment including the extensive fibrotic reaction. This chapter highlights the potential to therapeutically target PSCs which has the potential to decrease the production of fibrous tissue, slow down tumor progression and increase the delivery of chemotherapeutic agents to the tumor.

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