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Microenvironment in Determining Chemo-resistance in Pancreatic Cancer: Neighborhood Matters

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Abstract

Every year, nearly 300,000 people are diagnosed with pancreatic cancer worldwide, and an equivalent number succumb to this disease. One of the major challenges of pancreatic cancer that contributes to its poor survival rates is the development of resistance to the standard chemotherapy. Heterogeneity of the tumor, the dense fibroblastic stroma, and the aggressive biology of the tumor all contribute to the chemoresistant phenotype. In addition, the acellular components of the tumor microenvironment like hypoxia, stress pathways in the stromal cells, and the cytokines that are secreted by the immune cells, have a definitive role in orchestrating the chemoresistant property of the tumor. In this review, we systematically focus on the role played by the different microenvironmental components in determining chemoresistance of pancreatic tumors.

Introduction

Worldwide, almost 300,000 people are detected yearly with pancreatic cancer and an equivalent number succumb to this disease. In the US alone, the predicted number of pancreatic cancer cases in 2016 is estimated to be more than 53,000, and it is predicted that almost 41,000 of these will succumb to this disease (www.cancer.org). The 5-year survival is about 6 % in patients with pancreatic cancer and this figure has remained relatively unchanged over the past 25 years ¹. The majority of patients present with locally advanced or metastatic disease, and such individuals have a grim median survival of 6–10 months, and 3–6 months, respectively ². One of the major challenges that are responsible for this poor prognosis is the extreme chemoresistant phenotype of the tumor.

One of the main problems associated with chemotherapy has been that patient tumors with the same histology do not necessarily respond identically to the same therapeutic regimen. Identifying the presence of resistance mechanisms and other determinants for drug sensitivity, in order to classify tumors into response categories, has been an ongoing research effort. Recent studies have shown that the heterogeneity within the tumor contributes

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significantly to the tumor response to any kind of chemotherapy^{3, 4}. In addition to the heterogeneity within the tumor sub-types, current literature on pancreatic tumor biology has established beyond doubt that the tumor is a milieu of a large number of independent components that comprise its “microenvironment”. These include the fibro-inflammatory stroma, the secreted extracellular matrix, the infiltrating immune population, as well as the tumor initiating cell population⁵⁻⁹. Each of these components has a distinct role in conferring chemoresistance to the pancreatic tumor. The current review is focused on understanding how the tumor microenvironment may be instrumental in mediating the chemo-resistant property of pancreatic tumor (Figure 1).

Stroma as a physical barrier to drug delivery

The stroma has remained a controversial element in the pancreatic tumor microenvironment. To date, it has not been elucidated whether it plays a significant role in conferring chemoresistance. The fibro-inflammatory stroma develops along with the tumor. The primary components of the stroma are the fibroblasts, the immune cells, and the extracellular matrix (ECM) secreted by the cellular components. As the stroma develops, the interstitial pressure of the stromal cells and the ECM constrict the blood vessels, thereby not only reducing flow of oxygen and nutrients but also chemotherapeutic compounds^{10, 11}.

However, targeting the stroma has not yielded significant benefit in terms of the survival in pancreatic cancer patients. A study using the spontaneous pancreatic cancer mouse model (the *Kras*^{G12D}; TP53 mutated mice or KPC mice)¹² proposed targeting the hyaluronan, a primary component of the ECM, in the pancreatic cancer stroma can relieve the pressure on the blood vessels thereby improving survival. This resulted in use of PEGylated hyaluronidase in combination with gemcitabine in the KPC mouse model. Though this study yielded promising results in mouse models, the Phase I clinical trial using this compound was terminated in 2014, as it did not offer any substantial benefit to patients compared to the current standard of care.

Intercepting stromal signaling pathways have been developed as another strategy to overcome the physical barrier posed by this tumor component. Of these, Sonic Hedgehog (SHH) signaling has been shown to be restricted to the stromal compartment¹³. Thus, pharmacologic inhibition of the SHH pathway was hypothesized to have a positive impact on gemcitabine delivery, by reducing the desmoplastic stroma. However, though use of a smoothed inhibitor (IPI-926) with gemcitabine caused depletion of tumor stroma and resulted in increased micro-vessel density in animals, these inhibitors failed to improve survival in the recruited patients in clinical trials in 2012¹⁴.

Later, a study by Rhim *et. al* in 2014 demonstrated that the role of stroma in pancreatic cancer may be restraining, and thus, inhibiting it particularly at an early stage of tumor development would likely be detrimental for survival and this would result in extensive metastasis of the cancer¹⁵. In a parallel study by Ozdemir *et. al.* further showed that myofibroblast depletion in the tumor microenvironment led to extensive remodeling of the tumor ECM, with a significant decrease in tumor tissue stiffness and total collagen content. Interestingly, in this study, treatment with gemcitabine following myofibroblast depletion

did not lead to increased survival¹⁶. The study further showed that the most dramatic impact of myofibroblast depletion was on the composition of the immune infiltrate in the tumor microenvironment. The interplay between cancer-associated fibroblasts and immune cells has long been recognized as a major contributor of cancer development^{17, 18}. This study also demonstrated that the immune response (innate and adaptive) associated with pancreatic adenocarcinoma (PDAC) was significantly impaired when fibrosis was reduced starting at the PanIN stage or at the PDAC stage.

The debate of whether the stroma is beneficial for pancreatic tumor progression and resistance to therapy continues. In keeping with this concept, it can be argued that while depleting stroma in a tumor will result in relieving the interstitial pressure on the blood vessels and promote drug delivery to the tumor, the tumor cells need to be obliterated as well in order to prevent their extravasation and spread to a distant location. This hypothesis is supported by a recent publication on two experimental drugs: one of the studies published in 2015, showed that ormeloxifene, a non-hormonal, non-steroidal oral contraceptive to be effective in reducing desmoplasia as well as induce tumor cell death in pancreatic cancer^{19–21}. The other study, also from 2015, evaluated a water-soluble diterpene triepoxide, Minnelide against both patient tumor derived xenografts as well as the spontaneous KPC model for pancreatic cancer. Minnelide was also able to decrease desmoplasia, increase drug delivery and induce tumor cell death at the same time²². Early studies using Minnelide in multiple animal models have also shown that it prevents metastasis, which supports the hypothesis that the depletion of stromal component in pancreatic cancer will only be effective in tumor regression if the anti-stromal compound is used in combination with anti-tumor and anti-metastatic drugs.

Pancreatic Stellate Cells conferring chemoresistance

Pancreatic Stellate Cells (PSCs) are a quiescent population of cells within the pancreas, which are an integral part of the tumor microenvironment. In a normal, disease-free state, these cells express desmin, glial fibrillary acidic protein (GFAP), and store cytoplasmic vitamin A-containing lipid droplets^{23–25}. The vitamin A droplets make them a distinct cell type that is different from the normal pancreatic fibroblast. Upon onset of disease, accumulation of reactive oxygen species, cytokines, and growth factors secreted by injured cells, activate PSCs stimulating them to differentiate into a myofibroblast like phenotype^{26, 27}.

hPSC secretions have been shown to confer a chemoresistant cancer cell phenotype by (i) suppressing H₂O₂-induced apoptosis and increased survival of pancreatic cancer cells²⁸; and (ii) decreased pancreatic cancer cell sensitivity to gemcitabine and radiation therapy²⁹. In addition, pancreatic cancer cells cultured with ECM proteins, produced by PSCs, promoted resistance to 5-fluorouracil (5-FU), cisplatin, and doxorubicin³⁰. However, a limitation in the field is that no studies have examined the influence of PSCs on proteins, which protect tumor cells against chemotherapy agents (for example multi-drug resistant drug transporters). Further, recent work by Liu, *et al* have shown that periostin, exclusively overexpressed by the pancreatic stellate cells, confer resistance to gemcitabine in pancreatic cells and tumors³¹. In an independent study, it was also reported that PSCs promoted the

expression of Hes1 in pancreatic cancer cells, which in turn contributed to their resistance to chemotherapy³². Additionally, the SDF1/CXCR4 axis has recently emerged as an important regulator of tumor-stroma interaction. SDF1 is secreted by the PSCs, while its receptor CXCR4, is secreted by the pancreatic cancer cells. A study by Zhang, *et al* revealed that PSCs promote the chemoresistance of pancreatic cancer cells to gemcitabine by paracrine SDF-1 α /CXCR4 signaling-induced activation of the intracellular FAK-AKT and ERK1/2 signaling pathways and a subsequent IL-6 autocrine loop in the cancer cells³³.

It is important to note that in addition to the impact which stellate cells have on pancreatic cancer chemoresistance, it is now well established that the immune cells also impact tumor progression and chemoresistance³⁴. Immune cells within the tumor microenvironment activate PSCs, which may further potentiate the effect of stellate cells on chemoresistance.

Pancreatic Stellate Cells and radio-resistance

Almost ~40% of pancreatic cancer patients undergo external beam radiation therapy (EBRT)³⁵. Radiation therapy plays a significant role in locally advanced pancreatic cancer, particularly when patients do not have distant metastasis following a chemotherapy regimen. However, clinical trials testing the ability of radiation therapy to extend progression-free survival or increase overall survival either alone or in combination with chemotherapy, have not shown very conclusive results^{35, 36}. In addition, only 20% of pancreatic primary tumors show any significant response to radiation³⁵.

The molecular pathways contributing to apparent resistance to ionising radiation (IR) in PC remain poorly understood^{37, 38}. However, recent research has shown that pancreatic stellate cells may play a significant role in contributing to the radio-resistance of the tumor. Radioresistance conferred by the stellate cells was shown to be mediated by β -Intergrin-FAK signaling in the tumor cells³⁹. Since this initial report, several studies have highlighted the radio-protective role played by the PSCs on the tumor. Recent studies by Al-Assar et al in 2014⁴⁰ as well as in 2016⁴¹ showed that the presence of PSCs in a co-culture with pancreatic cancer cells protect the cancer cells from radiation induced cell death⁴². The contribution of stellate cells to radioprotection of pancreatic tumor is an upcoming and developing field and much remains to be done in this respect in order to gain a complete insight into the molecular mechanisms that may be involved in the process.

Hypoxic microenvironment as a factor for chemoresistance

As the tumor progresses, the developing stroma exerts pressure on blood vessels, resulting in constriction and increasing hypoxic niches in the tumor. However, this hypoxia does not lead to increased angiogenesis in pancreatic adenocarcinoma like pancreatic neuroendocrine tumors^{14, 43}. Instead, this hypoxia activates multiple signaling pathways that may contribute to chemoresistance.

It is known that a hypoxic environment exists both in pancreatic cancer cells as well as in surrounding PSCs. A study by Masamune showed that hypoxia induced migration, type I collagen expression, and vascular endothelial growth factor (VEGF) production in PSCs, suggesting its pro-fibrogenic and pro-angiogenic responses in these cells⁴⁴. Similarly, a

study by Erkan, *et al* showed that in the peritumoral stroma, PSCs contribute to the fibrotic-hypoxic milieu through abnormal extracellular matrix deposition, and by amplifying endostatin production of cancer cells⁴⁵.

Low oxygen content in the tumor results in stabilization of the Hypoxia Inducible Factor 1 (HIF1A). HIF1A is the central node that mediates activation of a number of different signaling pathways that alter metabolic pathways, induce invasiveness, promote chemoresistance, and lead to a poor prognosis of the patient ⁴⁶. HIF has been reported to participate in drug and radiotherapy resistance in cancer treatment. Radiotherapy (ionizing radiation) causes DNA damage directly and indirectly. Ionizing radiation and some chemotherapeutic drugs cause intracellular/tissue water ionizing to produce free radical (to impair DNA). Lack of oxygen reduces the cytotoxic effects of drugs and radiotherapy directly. Therefore, areas of hypoxic tumor tissue are more resistant to treatment and are associated with a poor clinical prognosis ⁴⁷.

HIF1A is a tightly regulated protein in the tumor cell that is degraded under normal oxygen content. However, upon hypoxic stress, HIF1A accumulates and compensates for low oxygen by increasing glycolysis and glucose uptake in the cells. This in turn results in the switch of cellular metabolism from oxidative phosphorylation to aerobic glycolysis, or the Warburg effect. The Warburg effect is not only a critical cellular metabolic adaptation to cyclic hypoxia, it is also an obviously beneficial trade-off for cancer cells to increase chemoresistance, mutation rate, and invasion/metastatic ability ⁴⁸. The increase in glycolytic metabolism also results in the production of lactate, which results in the acidification of the extracellular environment. The resulting extracellular acidification coupled with HIF-1 α -induced expression of carbonic anhydrases causes a significant change in the pH ratio between the intracellular and extracellular environment. This pH shift decreases the passive absorption of many drugs that would otherwise accumulate at a greater concentration within the cell^{49, 50}.

Despite their roles in energy biogenesis, mitochondria also play an important role in the control of cell death. Mitochondria regulate cell death pathways not only through control of intrinsic apoptotic pathways but also the generation of reactive oxygen species ⁵¹. It has been shown that chemotherapy induced tumor cell death is mediated through the generation of reactive oxygen species. The generation of reactive oxygen species is suppressed in tumor cells under hypoxia by HIF1A thus conferring the drug resistance of tumor cells ^{52, 53}.

Microenvironment niches promote stemness and chemoresistance within a tumor

Recent research in the field has shown that the enrichment of cancer stem cells depends on the microenvironment niches⁵⁴ In pancreatic cancer, in addition to regulating altered metabolism in order to combat hypoxic stress in the tumor, HIF1A also regulates stemness in the tumor microenvironment by upregulating self-renewal genes in the tumor ^{6, 55–57}. Thus, the hypoxic niche in the tumor microenvironment is one of the major driving forces for the tumor initiating population within it. In addition to hypoxia, the stromal cells can also

enrich for tumor initiating cells. Recent work by Lonardo *et al* have also shown that pancreatic stellate cells are able to provide a niche for the cancer stem cells as well⁵⁸.

Tumor initiating cells (TICs) are a quiescent population within a tumor that escape chemotherapeutic drug induced cell death by either having an increased drug efflux property or altered proliferative index. While standard chemotherapy targets rapidly dividing cell population, the TICs, being quiescent, remain unaffected by these drugs. A number of recent researches have been focused on understanding the mechanism by which this rare population within a tumor escapes chemotherapeutics drugs^{56, 59–64}.

One of the characteristics of TICs is an increased drug efflux. These cells have an abnormally high activity of ATP driven xenobiotic transporters like the ABC transporter family, which help in keeping the intracellular drug concentration low and thereby help the cell to evade the cytotoxic effects of the drugs. Increased activity of ABC transporters is typically fueled by increased metabolic needs of the tumor cells. This further stresses the role of hypoxia in the tumor microenvironment mediating altered metabolic needs of the tumor cells resulting in increased efflux activity of ABC transporters in the TICs^{65–67}.

Inflammation, Immune cells and chemoresistance

Role of inflammation and inflammatory molecules in progression of pancreatic cancer is well known^{8, 17, 68}. The involvement of these in regulating the chemoresistant phenotype of the pancreatic tumor are becoming apparent by recent studies^{69–71}. As the pancreatic tumor progresses, the cross talk between the different cell types in the microenvironment get actively involved in recruiting the fibro-inflammatory stroma. Paracrine signaling, involving the cytokines and chemokines from the infiltrating cells, facilitate a tumor stroma interaction, resulting in formation of a reactive stroma that contributes to the chemoresistant nature of the tumor.

Inflammation within the pancreatic tumor microenvironment has been mechanistically linked to tumor progression and chemoresistance through NF- κ B, IL-6, toll-like receptor and TGF- β signaling pathways^{72, 73}. Unlike ovarian and colorectal cancers, survival gains from immune cell infiltration into the tumor microenvironment have not been conclusively demonstrated in pancreatic cancer^{74, 75}. In a recent study, Delitto *et al* examined the inflammatory milieu present in the pancreatic cancer microenvironment from 36 freshly resected tumor specimens using a forty-one-item panel of cytokines, chemokines and growth factors. This study showed that among others, increased intratumoral IL-8 concentrations were associated with larger tumors and poor differentiation; the administration of neoadjuvant chemotherapy was associated with reduced IL-8 concentrations⁸. Similarly, elevated levels of pro-inflammatory cytokines IL-1 β and TNF α were associated with a poor histopathologic response to neoadjuvant therapy⁸. However, the molecular mechanisms of how this is instrumental in conferring a chemoresistant phenotype to the tumor needs to be determined by future studies.

Anti-tumor necrosis factor-alpha (TNF- α) antibodies have promising effects in a number of PDAC pre-clinical models. Inhibition of TNF- α has also been shown to synergize with

chemotherapy in PDAC and result in a better pre-clinical response by killing tumor cells as well as diminishing desmoplasia and inflammation in PDAC tumor stroma⁷⁶.

Another predominant component of the microenvironment constituting a “niche” are the immune cells. Together with the stroma, these cells play a significant role in determining the chemoresistant phenotype of the pancreatic tumor. The immune system monitors and eliminates pathogens as well as developing tumors. Early studies of hematological malignancies suggested that tumor-associated macrophages (TAMs) can promote chemoresistance by directly interacting with malignant cells within the tumor microenvironment⁷⁷. It is now clear that TAMs can influence the response of cancer cells to chemotherapy in the context of a process known as environment-mediated drug resistance^{78, 79}.

A study by Amit *et al* showed that TAMs stimulate chemoresistance by promoting the expression of CDA, the enzyme responsible for the inactivation of gemcitabine, by cancer cells. Using TAM-conditioned medium, the study showed a decrease in apoptosis of malignant cells exposed to gemcitabine. The co-culture of TAMs and cancer cells had similar effect on gemcitabine-induced apoptosis compared to exposure of the latter to TAM-conditioned medium, suggesting that one or more soluble factors secreted by TAMs mediate(s) this effect. In line with this finding, macrophage-depleted mice were more sensitive to the antineoplastic effects of gemcitabine than their wild-type counterparts. This study thus demonstrated for the first time that macrophages could increase the resistance of cancer cells to chemotherapy through the upregulation of CDA, an intracellular enzyme which catabolizes the active form of gemcitabine⁸⁰.

Conclusion

Pancreatic cancer poses a major therapeutic challenge. So far, only small progress has been in the diagnosis and management of patients with this disease. The recent discoveries in the field of genetics, biology, metabolism and immunology of pancreatic cancer have created new opportunities to develop novel approaches for earlier diagnosis and more effective treatment. More research is clearly needed to improve our biological understanding. It remains to be demonstrated whether therapeutic targeting of the different components of the tumor microenvironment, either alone or in combination with conventional treatments, will improve the outcome of patients with pancreatic cancer in the near future. Thus, understanding the role of microenvironment in tumor progression as well as its response to intervention is of extreme importance for development of novel therapy against this devastating disease.

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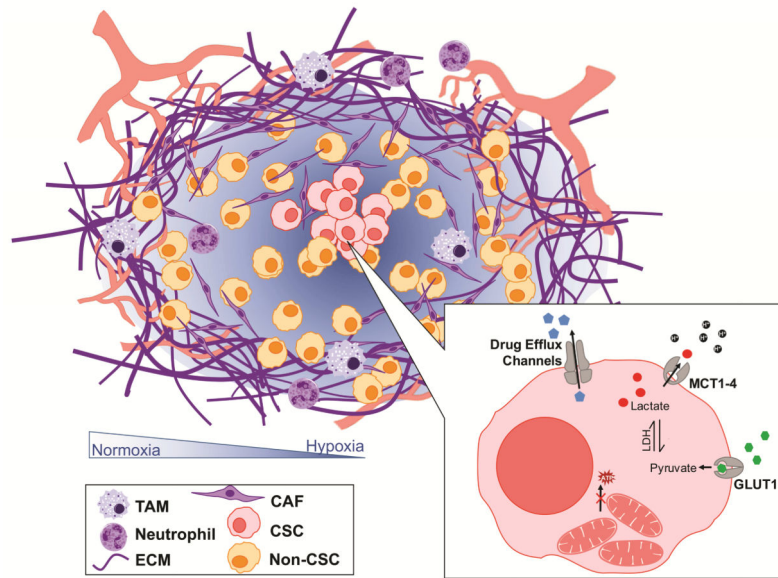


Figure 1.

A schematic displaying the role of the microenvironment in pancreatic adenocarcinomas (TME). The TME includes the immune cells (tumor associated macrophages (TAM) and neutrophils); stroma (extra cellular matrix (ECM) and cancer associated fibroblasts (CAF)); cancer stem cells (CSC) and non-CSC; in a hypoxic environment. Further, CSCs contain inactive mitochondria, higher levels of drug efflux channels, increased glucose uptake and lactate export.