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Stromal heterogeneity in pancreatic cancer and chronic pancreatitis

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ABSTRACT

Background/objectives: An abundant stromal reaction is a hallmark of pancreatic ductal adenocarcinoma (PDAC) and chronic pancreatitis (CP). The cells mainly responsible for the stromal reaction are activated pancreatic stellate cells (PSCs). Despite their crucial role, PSCs are not well characterized. PSCs share characteristics with the better-known hepatic stellate cells (HSCs). The aim of this study was a detailed analysis of PSCs in PDAC and CP.

Methods: Whole-slide specimens of CP (n = 12) and PDAC (n = 10) were studied by histochemistry and immunohistochemistry. The stroma was evaluated using Movat's pentachrome stain. PSCs were tested by immunohistochemistry for PSC markers (α-SMA, CD34, desmin, NGFR, SPARC and tenascin C) and HSC markers (α-crystallin B, CD56, NGF, NT-3, synaptophysin and TrkC). Alpha-SMA, tenascin C, SPARC and NT-3 staining were verified on tissue micro arrays (TMAs) from a well-characterized cohort of 223 PDAC patients. PSCs isolated from human PDAC and CP tissue samples as well as HSCs were evaluated by immunofluorescence.

Results: While the stroma of CP cases was characterized by a collagen-rich fibrosis, PDAC stroma displayed higher mucin content ($p = 0.0002$). PSCs showed variable expression of tested markers. In PDAC samples, staining of most markers was found around tumor complexes, while CP samples showed a greater variety of localizations. Alpha-SMA staining correlated with collagen-rich fibrosis ($p = 0.012$), while NT-3 staining correlated with mucin-rich stroma ($p = 0.008$). A peritumoral staining was confirmed for α-SMA, tenascin C, SPARC and NT-3 in the PDAC TMA cohort (n = 223). In a subgroup of patients with pancreatic head tumors and UICC 2009 IIB (n = 144), α-SMA staining intensity was a prognostic factor for overall survival at uni- and multivariate analysis ($p = 0.036$ and $p = 0.002$).

Conclusions: The close similarities between PSCs and HSCs were confirmed. Heterogeneous expression patterns of the tested markers might reflect different levels of activation or differentiation, or even multiple subpopulations of PSCs. Survival analysis suggests an impact of stromal composition on survival.

1. Introduction

An excessive stromal reaction is a characteristic feature of both pancreatic ductal adenocarcinoma (PDAC) and chronic pancreatitis (CP). In CP, the formation of an abundant stroma results in an eventual loss of exocrine and endocrine pancreatic function [1]. While some data imply that the stroma can have a protective effect in PDAC [2,3], many findings suggest that the stromal reaction contributes significantly to the aggressiveness and therapy resistance that characterizes PDAC [4–6].

Pancreatic stellate cells (PSCs) are established as key effectors of the stromal reaction in CP and PDAC [1,4,7]. They are resident cells of the healthy exocrine pancreas, where they comprise about 4-7% of the total cell mass and normally exist in a quiescent state distinguished by an angular shape and an abundance of cytoplasmic lipid droplets containing vitamin A [8]. The main function of PSCs is to regulate the turnover of extracellular matrix (ECM) proteins in order to ensure the maintenance of a normal stroma composition. For this purpose, PSCs are able to synthesize ECM

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proteins as well as matrix metalloproteinases and their tissue inhibitors [7,9]. Upon tissue injury, like inflammation or carcinogenesis, but also during *in vitro* cultivation, PSCs transform into a star-shaped myofibroblast-like phenotype and synthesize and secrete excessive amounts of ECM proteins as well as growth factors and cytokines [4,10]. The microfilament forming cytoskeletal protein α-smooth muscle actin (α-SMA) is a well-established marker for activated PSCs [8], or rather, for PSCs that undergo transdifferentiation into an activated phenotype [11].

Several *in vitro* and *in vivo* studies have shown that PSCs interact reciprocally with pancreatic cancer cells (PCCs): PCCs increase proliferation, ECM synthesis and migration in PSCs [12,13]. Vice versa, PSCs increase proliferation, migration as well as invasion and inhibit apoptosis in PCCs [13]. Findings like these strongly support the general hypothesis that the significance of the stroma in chronic inflammatory diseases and cancer runs much deeper than only providing a physical framework that harbors inflammatory cells or tumor cells.

However, despite their pivotal role in two major pancreatic diseases, PSCs lack an in-depth characterization regarding their origin, immunophenotype and function, partly due to their relatively late discovery, isolation and cultivation [8,14,15], but also because research on cancer-supporting stromal cells had been sidelined by the focus on cancer cells themselves. Nevertheless, it is known that PSCs share many characteristics with their well-characterized counterparts in the liver, hepatic stellate cells (HSCs), and that both cell types are clearly distinguished from ordinary fibroblasts [16]. Comparable to the role that PSCs play in the pancreas, HSCs are the main contributors to liver fibrosis, among other functions [17].

The aim of this study was a detailed immunohistochemical characterization of PSCs in cases of PDAC as well as cases of CP of various etiologies, including alcohol-induced chronic pancreatitis, autoimmune pancreatitis type 1 and type 2 and hereditary pancreatitis. A similar approach has previously been used for the characterization of HSCs [18]. Using the known analogy between PSCs and HSCs, this study did not only investigate established and suggested PSC markers like α-SMA, CD34, desmin, NGFR, SPARC and tenascin C, but also known HSC markers like α-crystallin B, CD56, NGF, NT-3, synaptophysin and TrkC. While all these markers are linked to stellate cells, they are very different in their distribution and function. Alpha-SMA (α-smooth muscle actin) is a cytoskeletal protein responsible for cell structure and motility and a well-established marker for activated stellate cells [8,19]. Similarly, the intermediate filament protein desmin and the cell surface glycoprotein CD34, which is expressed on haematopoietic precursors and endothelia, have been established as stellate cell markers [8,18,20,21]. SPARC (secreted protein acidic and rich in cysteine; osteonectin) and tenascin C are both ECM proteins involved in cell-ECM interactions, especially in the context of tissue remodeling during inflammation and carcinogenesis [22,23]. NGF (nerve growth factor) and NT-3 (neurotrophin-3) belong to the protein family of neurotrophins, which promote the survival, growth and differentiation of neurons [24]. NGF and NT-3 both bind to the low-affinity universal neurotrophin receptor NGFR (nerve growth factor receptor), while TrkC (tropomyosin receptor kinase C) is the main receptor of NT-3 [25,26]. The expression of NGF, NGFR and TrkC has been linked to perineural invasion in pancreatic cancer [27–29]. CD56, also known as NCAM (neural cell adhesion molecule), is known to be expressed by numerous neural and neuroendocrine cells [30], plays a role in cell-cell and cell-ECM adhesion and has signaling properties [31]. Another common neuroendocrine marker evaluated in this study is synaptophysin, which seems to be important for synaptic vesicle exo- and endocytosis [32]. Alpha-crystallin B is a protein of the small heat shock protein super family expressed in many different tissues, but is especially known for its expression in peripheral nerves [33].

2. Material and methods

2.1. Cell culture

PSCs and HSCs were isolated from patients who underwent pancreatic (n = 5, two PDAC and three CP) or hepatic resection (n = 1) at the Klinikum rechts der Isar, Munich, Germany, via the outgrowth method, as previously described [34]. The use of human tissue samples was approved by the local ethics committee at the Klinikum rechts der Isar, Munich, Germany (5510/12). The stellate cell phenotype was confirmed by immunostaining for α-SMA [8,11]. PSCs and HSCs were maintained in a 1:1 solution of Ham's F12 nutrient medium and low-glucose DMEM (1 g/L) supplemented with 20% FBS, 1% penicillin/streptomycin and 1% amphotericin B (all reagents from Gibco®, Darmstadt, Germany) in humidified air at 37 °C and 5% CO_2.

2.2. Immunofluorescence

Antibodies are listed in Table S1. Cells seeded onto coverslips were rinsed with PBS and fixed with chilled methanol for 5 min at room temperature (RT). Between all following steps, cells were rinsed with TBS buffer (50 mM TRIS, 138 mM NaCl, 2.7 mM KCl, pH 7.6) for 5 min three times. Cells were permeabilized with a solution of TBS containing 0.25% Triton X100 for 5 min and incubated with blocking solution (TBS containing 10% goat serum and 0.1% Triton X100) for 30 min at RT, before being incubated with the primary antibody appropriately diluted in blocking solution overnight in a humidified chamber at 4 °C. Cells were then incubated with the Alexa Fluor conjugated secondary antibody diluted in blocking solution (488 goat anti-mouse or 647 goat anti-rabbit, 1:200 each; Invitrogen GmbH, Karlsruhe, Germany) for 60 min and counterstained with Hoechst 33342 (0.5 μg/mL) for 5 min at RT. After being rinsed with TBS for 5 min twice and with distilled water for 5 min once, cells on coverslips were placed onto microscopic glass slides provided with a drop of anti-fade mounting medium (Vectashield, Loerrach, Germany). Slides were examined under a fluorescence microscope, images were obtained with the Zeiss AxioVision software (Carl Zeiss AG, Jena, Germany). Negative controls were performed omitting the primary antibodies, U251 glial cells (ATCC, Manassas, USA) were used as positive controls for neural marker proteins.

2.3. Tissue collection

Tissue samples of whole sections were obtained from patients who underwent pancreatic resection due to either PDAC (n = 10) or CP (n = 12) at the Department of Surgery of the University Hospital of Heidelberg, Germany. Tissue samples were fixed in 4% formalin and subsequently routinely embedded in paraffin. The use of human tissue samples was approved by the local ethics committee at the University Hospital of Heidelberg, Germany (study number 301/2001).

Tissue samples for tissue micro arrays (TMAs) were obtained from patients who underwent pancreatic resection for PDAC (n = 223) at the Department of Surgery of the University Hospital of Duesseldorf, Germany. The samples were fixed in 4% formalin and paraffin-embedded in blocks. Subsequently, three tumor samples per case (two samples from the tumor center, one from the periphery) with a 1-mm core size were selected and assembled into the TMA (Manual Tissue Arrayer MTA-1, Beecher Instruments, Inc., Sun Prairie, WI, USA). The use of human tissue samples was

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