Pancreatic Carcinoma Cells Induce Fibrosis by Stimulating Proliferation and Matrix Synthesis of Stellate Cells

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Background & Aims: **Tumor desmoplasia is one of the representative histopathologic findings in ductal pancreatic adenocarcinoma. The aims of this study were to examine the cellular and molecular mechanisms of fibrogenesis associated with pancreatic adenocarcinomas.** ***Methods:*** **Immunostainings were performed with human pancreatic adenocarcinomas (n = 27) and tumors induced in nude mice (n = 36) by subcutaneously injecting MiaPaCa2, Panc1, and SW850 with and without pancreatic stellate cells. Matrix-producing cells were isolated from pancreatic adenocarcinomas and compared with pancreatic stellate cells isolated from tissue of chronic pancreatitis. Paracrine stimulation of pancreatic stellate cells by carcinoma cells was studied regarding matrix synthesis (collagen and c-fibronectin on protein and messenger RNA level) and cell proliferation (bromodeoxyuridine incorporation).** ***Results:*** **High numbers of desmin and α-smooth muscle actin–positive cells were detected in 26 of 27 pancreatic adenocarcinomas. Intense fibronectin and collagen stainings were associated with these cells. By using cytofilament stainings, gene expression profiling, and morphological examinations, the matrix-producing cells obtained by the outgrowth method from pancreatic adenocarcinomas were identified as pancreatic stellate cells. Supernatants of MiaPaCa2, Panc1, and SW850 cells stimulated proliferation and collagen type I and c-fibronectin synthesis of cultured pancreatic stellate cells. Preincubation of the carcinoma cell supernatants with neutralizing antibodies against fibroblast growth factor 2, transforming growth factor β1, and platelet-derived growth factor significantly reduced the stimulatory effects. Subcutaneous injection of carcinoma cells and pancreatic stellate cells induced fast-growing subcutaneous fibrotic tumors in nude mice. Morphometric analysis of carcinoma cells (cytokeratin stainings) showed a high density of carcinoma cells in these tumors.** ***Conclusions:*** **Pancreatic stellate cells strongly support tumor growth in the nude mouse model. The increased deposition of connective tissue in pancreatic carcinoma is the result of a paracrine stimulation of pancreatic stellate cells by carcinoma cells.**

Ductal adenocarcinomas of the pancreas are characterized by rapid progression, early metastasis, diagnosis at an advanced stage, and a limited response to chemotherapy and radiotherapy.[1,2] Tumor desmoplasia, a process in which fibrous tissue infiltrates and envelops neoplasms, is one of the representative histopathologic findings in ductal pancreatic adenocarcinomas (PAC).[3–7] In all cases of pancreatic ductal adenocarcinomas studied by Mollenhauer et al,[7] a remarkable increase in interstitial connective tissue (collagen type I and fibronectin) was observed. A similar distribution of collagens was observed in lymph node and liver metastases and in tumors xenografted into nude mice.[7] The mean collagen content in pancreatic cancer tissue and tumor-associated chronic pancreatitis is 3-fold higher than in normal pancreas, and there is no difference in the proportion of collagen types I, III, and V among alcoholic chronic pancreatitis, tumor-associated chronic pancreatitis, and pancreatic cancer tissue.[8]

Although pancreatic carcinoma cell (CC) lines are able to produce collagen types I, III, and IV and fibronectin, laminin, vitronectin, and undulin in vitro and in vivo,[9] most reports indicate that the fibrotic extracellular matrix (ECM) associated with PAC is produced by stromal cells.[6,10] In situ hybridization studies using human pancreatic cancer tissues localized transcripts coding for collagen types I and III to spindle-shaped cells, whereas transcripts for matrix metalloproteinase 2, matrix metalloproteinase 9, tissue inhibitor of metalloproteinase 1,

Abbreviations used in this paper: BrdU, bromodeoxyuridine; CC, carcinoma cell; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FCS, fetal calf serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; HSC, hepatic stellate cell; PAC, pancreatic adenocarcinoma; PDGF, platelet-derived growth factor; PSC, pancreatic stellate cell; RT-PCR, reverse-transcription polymerase chain reaction; α-SMA, α-smooth muscle actin; SN, supernatant; TSA, tyramide signal amplification.

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and tissue inhibitor of metalloproteinase 2 were found in both stromal and tumor cells.[11] In liver, activated myofibroblasts derived from hepatic stellate cells (HSCs) are responsible for an increased peritumor collagen production in hepatocellular carcinomas[12–14] and in metastases of colorectal tumors.[14] In addition, in experimental hepatocarcinogenesis, the number of activated HSCs increased in fibrous septa around and within dysplastic and carcinomatous nodules, thus suggesting that HSC activation may result from direct stimulation by factors released from dysplastic hepatocytes.[15]

Since the first reports on the identification, isolation, and characterization of pancreatic stellate cells (PSCs)[16,17] numerous in vivo and in vitro studies have provided strong evidence of a central role for PSCs in fibrogenesis associated with acute and chronic pancreatitis (see reviews[18,19]). PSCs share homologies to HSCs, including storage of retinyl palmitate, retinol esterification, expression of the cytofilaments vimentin and desmin, and phenotypic transition to an α-smooth muscle actin (α-SMA)–positive matrix–producing myofibroblast-like cell.[16,17,20,21] Transforming growth factor (TGF)-β1 and tumor necrosis factor α stimulate the change in the cell phenotype.[22] In addition, we have shown that platelet-derived growth factor (PDGF) is the most effective mitogen and that fibroblast growth factor (FGF)-2 and TGF-β1 are fibrogenic mediators that stimulate ECM synthesis of the activated phenotype of cultured rat and human PSCs.[17,22] Supernatants (SN) of activated macrophages[23] and platelet lysate[24] also stimulated proliferation and matrix synthesis of cultured PSCs. Others have shown that acetaldehyde, which is produced primarily in the liver by ethanol oxidation, and ethanol itself directly stimulate collagen synthesis of cultured PSCs.[25] In addition to these cell-culture data, it was shown that PSCs are the cell type responsible for connective tissue synthesis in experimental pancreatic fibrosis[21,26,27] and in human chronic alcoholic pancreatitis.[21,28] Recently Yen et al[29] described a pronounced increase in the numbers of α-SMA–positive cells in PAC. The authors suggested that these cells might be activated stellate cells producing the connective tissue surrounding and infiltrating PAC. In addition, very recently Apte et al[30] identified activated stellate cells in fibrotic areas of pancreatic cancers by staining for desmin, α-SMA, and glial acidic fibrillary protein. They also suggested that interactions between tumor cells and stromal cells (PSCs) may play an important role in the pathobiology of pancreatic cancer.[30]

To date, the role of the ECM in pancreatic cancer progression or restriction has not been defined. A role of the microenvironment playing a decisive role in tumor growth and metastatic spreading has been shown in a variety of experimental systems.[31,32] In small-cell lung cancer, ECM proteins protect cancer cells against apoptosis and accelerate cancer growth,[33] thus indicating that, at least in this tumor type, the stroma seems to be beneficial for the tumor. Another recent report showed that an interaction between pancreatic CCs and fibroblasts contributed to the development of chemoresistance.[34] In addition, Buchholz and coworkers[35] hypothesized recently that pancreatic cancer cells might stimulate PSCs to create a reactive microenvironment favoring invasive growth of the tumor.

The objective of this study was to examine the cellular and molecular mechanisms of fibrogenesis associated with human PAC. To examine this issue, we (1) performed immunohistology of human PACs, (2) isolated and characterized PSCs from PACs, (3) studied paracrine stimulation of cultured PSCs by CC-SNs, and (4) induced subcutaneous tumors into nude mice by injecting CCs with PSCs. We showed that the increased deposition of connective tissue in pancreatic carcinoma is the result of a paracrine stimulation of PSCs by cancer cells. Furthermore, we conclude from our data that PSCs support tumor growth in the nude mouse model.

Materials and Methods

Reagents were purchased from the following sources: acetone from Merck (Darmstadt, Germany); bisbenzimide from Hoechst (Frankfurt, Germany); fetal calf serum (FCS), amphotericin B, anti–α-SMA, High Pure RNA Extraction Kit, LightCycler FastStart SYBR Green, and LightCycler DNA Master SYBR Green I from Roche (Mannheim, Germany); gelatin type B, ethidium bromide, bovine albumin fraction V, ethanol, L(+)-ascorbic acid, calf thymus DNA, yeast transfer RNA, and RedTaq DNA Polymerase from Sigma (Deisenhofen, Germany); L-glutamine and Superscript from GibcoBRL (Paisley, Scotland); Delfia Eu-labeled streptavidin, Delfia Eu-labeled anti-rabbit immunoglobulin G, and enhancement solution from PerkinElmer-Life Science-Wallac (Turku, Finland); rabbit anti-human collagen type I and biotin-labeled goat anti-human collagen type III from Chemicon International (Temecula, CA); rabbit anti-fibronectin from Dade-Behring (Marburg, Germany); goat polyclonal neutralizing anti-PDGF(AA), anti-PDGF(BB), and anti–FGF-2 and rabbit neutralizing anti–TGF-β1 from R&D Systems Inc. (Minneapolis, MN); anti-human collagen type I from Southern Biotechnology Associates (Birmingham, AL), poly-dA/dU homopolymer from Pharmacia LKB (Freiburg, Germany); Hybond-N membrane, Hybond-C extra membrane, and Random Priming Kit (RPN 1601Y) from Amersham-Buchler (Braunschweig, Germany); anti-bromodeoxyuridine (BrdU), biotinylated swine anti-rabbit, horseradish peroxidase (HRP) swine anti-rabbit, HRP rabbit anti-mouse, fluorescein isothiocyanate (FITC)-conjugated streptavidin, biotinylated rabbit anti-