Pancreatic Neuropathy and Neuropathic Pain—A Comprehensive Pathomorphological Study of 546 Cases

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Background & Aims: **Chronic pancreatitis (CP) and pancreatic adenocarcinoma (PCa) are characterized by intrapancreatic neural alterations and pain. Our aims were to: (a) Investigate whether neuropathic changes like pancreatic neuritis, increased neural density, and hypertrophy are phenomena only in CP or whether they are also evident in other pancreatic disorders as well, (b) study possible variations in neural cancer cell invasion among malignant pancreatic tumors, and (c) explore whether these neuropathic changes contribute to pain sensation.** ***Methods:*** **Neuropathic changes were studied in PCa (n = 149), in CP (n = 141), in pancreatic tumors (PTm) including serous/mucinous cystadenomas, invasive/noninvasive intraductal papillary mucinous neoplasias, benign/malignant neuroendocrine tumors, ampullary cancers (n = 196), and in normal pancreas (n = 60). The results were correlated with GAP-43 expression, tissue inflammation, pancreatic neuritis, neural invasion, fibrosis, desmoplasia, pain, and patient survival.** ***Results:*** **Increased neural density and hypertrophy were only detected in PCa and CP and were strongly associated with GAP-43 over expression and abdominal pain. The severity of pancreatic neuritis was strongest in PCa and was closely linked to changes in neural density and hypertrophy. The aggressiveness of neural cancer cell invasion was most prominent in PCa and was related to neuropathic changes, desmoplasia, and pain. Severe and enduring pain were strongly associated with poor prognosis in PCa patients.** ***Conclusions:*** **Enhanced neural density and hypertrophy are only typical features of CP and PCa among all investigated pancreatic disorders. Such neuropathic changes, including damage to nerves by inflammatory and/or cancer cells, seem to enhance and generate pancreatic neuropathic pain.**

Pancreatic adenocarcinoma (PCa) and chronic pancreatitis (CP) are characterized by prominent intrapancreatic neuropathic changes and abdominal pain. Neural alterations in PCa are typically represented by intra- and extrapancreatic perineural invasion of cancer cells and are observed in 71%–98% of pancreatic adenocarcinoma specimens.[1–3] Extrapancreatic nerve plexus invasion increases in parallel with the occurrence of intrapancreatic neural invasion, thereby causing abdominal pain, precluding curative resection, and limiting patient survival.[1,4–11] The cancer cells are not restricted to the periphery of nerves, but also penetrate the perineurium and become intimately associated with Schwann cells and axons in the endoneurium.[6] A corresponding phenomenon is observed in CP, characterized by severe damage on perineural sheaths of the intrapancreatic nerves with remarkable immune cell infiltration.[12] This phenomenon causes a local "pancreatic neuritis" which correlates with the severity of pain in CP patients.[13,14] Another typical neuropathic alteration in CP is the increased neural density and hypertrophy, characterized by distinctly enlarged and numerous intrapancreatic nerves, which are strongest in patients with severe fibrosis.[12,15,16] It has been puzzling whether intrapancreatic neural alterations are involved in pain generation and maintenance in pancreatic diseases. Little is known about the neuropathic changes in other pancreatic disorders, in particular concerning changes in neural density and hypertrophy, pancreatic neuritis, and neural cancer cell invasion.

For that reason, we have analyzed whether neuropathic changes, characterized by increased neural density and hypertrophy and pancreatic neuritis, are phenomena specific only for CP or whether they are common features of various subtypes of pancreatic tumors. Therefore, tissues from patients with PCa, CP, serous and mucinous cystadenoma, invasive and noninvasive intraductal papillary mucinous neoplasia, benign and malignant neuroendo-

Abbreviations used in this paper: AmpC, ampullary cancer; CP, chronic pancreatitis; IPMN, intraductal papillary mucinous neoplasia; IPMNi, invasive intraductal papillary mucinous neoplasia; MC, mucinous cystadenoma; Ni, neural invasion; NP, normal pancreas; NTb, benign neuroendocrine tumor; NTm, malignant neuroendocrine tumor; PCa, pancreatic adenocarcinoma; PTm, pancreatic tumors; SC, serous cystadenoma.

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crine tumors, ampullary cancer, and normal pancreas were investigated. Neuropathic alterations were correlated to the severity of tissue inflammation, pancreatic neuritis, the degree of neural cancer cell invasion, the severity of fibrosis and desmoplastic reactions. Possible differences in the neural invasion of cancer cells within the malignant pancreatic tumors, concerning neural invasion manifestation, severity, and frequency, were investigated in detail. To answer the question whether a feature like "neuropathic pain" exists in pancreatic disorders, subjective pain sensations of all patients were analyzed in all pancreatic disorders and correlated to neuropathic changes. Finally, the potential impact of intrapancreatic neuropathic alterations and pain on patient survival was determined.

Materials and Methods

Patients and Tissues

Pancreatic tissue samples were collected from patients following tumor resection: Pancreatic adenocarcinoma (PCa; $n = 149$), chronic pancreatitis (CP; $n = 141$) and other pancreatic tumors (PTm; n = 195) including mucinous (n = 12) and serous cystadenoma (n = 39), invasive intraductal papillary mucinous neoplasia (IPMNi; n = 40) and noninvasive intraductal papillary mucinous neoplasia (IPMN; n = 33), benign neuroendocrine tumor (NTb; n = 17) and malignant neuroendocrine tumor (NTm; n = 35), and ampullary cancer (AmpC; n = 19) (Supplementary Table 1; see supplementary Table 1 online at www.gastrojournal.org). Pancreatic endocrine tumors lacking criteria of malignancy (metastases and/or gross invasive behavior) were designated as "neuroendocrine benign." Normal pancreatic tissue samples were obtained from healthy organ donors (n = 60) whenever there was no suitable recipient available. The cancer stage of PCa, IPMNi, and AmpC was graded according to the international classification of the UICC 2002 (Supplementary Table 2 see supplementary Table 2 online at www.gastrojournal.org). The malignant pancreatic neuroendocrine tumors were classified according to the consensus conference of the European Neuroendocrine Tumor Society in Rome 2006, as follows: 1 patient with IIa, 6 with IIb, 17 with IIIb and 11 patients with stage IV. The study protocols were approved by the ethics committees of the University of Bern (Switzerland) and the University of Heidelberg (Germany). Tissue preservation was made as reported before.[16]

Reagents

Reagents purchased were as follows: Protein Gene Product 9.5 (PGP9.5) rabbit polyclonal antibody from Dako Cytomation (Hamburg, Germany) and GAP-43 mouse monoclonal antibody from Chemicon (Munich, Germany). Dako Envision system (Hamburg, Germany) was used for immunohistochemistry analysis. All other reagents were from Sigma–Aldrich Chemical Company (Taufkirchen, Germany).

Immunohistochemistry

Consecutive 3-μm paraffin-embedded tissue sections of all groups were analyzed for PGP9.5 and GAP-43 immunostaining using the Dako Envision system, as described previously.[17] PGP9.5 (1:1000) and GAP-43 (1:1000) antibodies were diluted in normal goat serum. Rabbit or mouse IgG (Dako) was used as negative control. Digital imaging was performed with the Zeiss AxioCam HR system (Carl Zeiss AG, Oberkochen, Germany).

Quantitative Analysis of the Nerve Number and the Total Nerve Area

Intrapancreatic nerves in tissue sections of normal pancreas (NP; n = 60), PCa (n = 149), CP (n = 141) and PTm (n = 195) were immunolabeled using the panneuronal marker PGP9.5. To quantify the total number of nerves per unit area (neural density) and the total nerve area (neural hypertrophy), the entire pancreatic tissue sections were scanned at high magnification ($\times$100) and reconstructed into a mosaic image using the Zeiss KS300 program, as demonstrated before.[16] In every microscopic cut-out of the mosaic, the PGP9.5 immunoreactive nerves were circumscribed. In pancreatic malignancies with endoneural invasion of cancer cells, only the nerve trunk without cancer cell invasion was included in the analysis. The number of nerves and the nerve area in the entire area of the investigated tissue sections were also assessed with the Zeiss KS300 program. Neural density was expressed as the total number of nerves within unit area (1 mm^2) of the investigated specimen. Accordingly, neural hypertrophy was expressed as the ratio of the total measured area of the nerves to the total number of nerves of the analysed section (μm^2).

Real-Time Light-Cycler Quantitative Polymerase Chain Reaction (QRT-PCR)

Extraction of mRNA from pancreatic tissues was prepared by automated isolation using the MagNa Pure LC instrument and isolation kit II. cDNA was prepared using the 1st-strand cDNA synthesis kit for RT-PCR (Roche Applied Science, Mannheim, Germany). QRT-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit. All primers were obtained from Search-LC (Heidelberg, Germany). The calculated number of specific transcripts were normalized, and expressed as the amount per μL of input cDNA, as described previously.[18]

Clinicopathological Analysis

Consecutive sections obtained from paraformaldehyde-fixed and paraffin-embedded pancreatic tissue from each patient were stained with hematoxylin and eosin for concomitant histomorphological examination. Histopathological analysis was performed in one representative tissue specimen from the center of each tumor by 2 independent observers blinded to patient diagnosis and QRT-PCR data, followed by resolution of any differ-