Upregulation of CD39/NTPDases and P2 Receptors in Human Pancreatic Disease

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Abstract:

Chronic inflammation, fibrosis, atrophy, malignant transformation and thromboembolic events are hallmarks of chronic pancreatic disease. Extracellular nucleotides have been implicated as inflammatory mediators in many pathological situations. However, there are minimal data detailing expression of ectonucleotidases and type-2 purinergic receptors (P2-R) in chronic pancreatitis and pancreatic cancer. We have therefore defined tissue distribution and localization of the CD39 family of ectonucleotidases and associated P2-R in human disease. Transcripts of ectonucleotidases (CD39, CD39L1) together with P2-R (P2X7, P2Y2 and P2Y6) are significantly increased in both chronic pancreatitis and pancreatic cancer. CD39 and CD39L1 are preferentially associated with the vasculature and stromal elements in pathological tissues. P2X7 mRNA upregulation was associated with chronic pancreatitis and heightened protein expression was found to be localized to infiltrating cells. P2Y2 was markedly upregulated in biopsies of pancreatic cancer tissues and expressed by fibroblasts adjacent to tumors. High tissue mRNA levels of CD39 significantly correlated with better long-term survival after tumor resection in patients with pancreatic cancer. Heightened expression patterns and localization patterns of CD39, P2X7 and P2Y2 infer associations with chronic inflammation and neoplasia of the pancreas. Our data suggest distinct roles for CD39 and P2-purinergic signaling in both tissue remodeling and fibrogenesis with respect to human pancreatic diseases.
Introduction:

Clinically important pancreatic diseases may be classified as acute pancreatitis (AP), chronic pancreatitis (CP) and pancreatic cancer (PaCa). Comparable biology and clinical features link these different entities. Recurrent attacks of acute pancreatitis, are believed to result in CP and this development is associated with increased risk of developing pancreatic cancer. (21, 23) Increased synthesis of extracellular matrix proteins (ECM) results in progressive fibrosis in CP and leads to dense desmoplastic reactions in pancreatic cancer. Fibrogenesis in CP is associated with acinar cell injury and necrosis, inflammation, activation of leukocytes, activation of pancreatic stellate cells (PSC), (1, 2) stimulated synthesis of extracellular matrix (ECM) and reduced matrix degradation with the ultimate matrix accumulation. (22) Release of nucleotides is increased following injury or cellular stress. (25)

Extracellular nucleotides regulate cell-cell communication via purinergic/pyrimidinergic P2-receptors (P2-R). There are two main families of extracellular nucleotide receptors: P2X are ligand-gated ion channels, permeable for Na⁺, K⁺, and also Ca²⁺ (subtypes P2X1-7); and P2Y are G protein-coupled receptors that initiate signal transduction coupled activation of phospholipase C, protein kinase C and adenylate cyclases. (5, 6, 13) Importantly, select P2Y receptors induce downstream effects acting through the inositol trisphosphate (IP3)-pathway, mediated by Ca²⁺. (10)

CD39 is an ectoenzyme, now referred to as nucleoside triphosphate diphosphohydrolase-1 (NTPDase1), that hydrolyzes extracellular nucleotides. (12) Vascular CD39 is the dominant endothelial ectonucleotidase and hydrolyzes both ATP and ADP in the plasma to AMP, ultimately to adenosine. (12) Another NTPDase associated with the vasculature is the cell-associated ecto-ATPase (CD39L1 or NTPDase2). (9)
NTPDases functionally interact with P2Y receptors. (12) For example, combinations of NTPDases have the capacity to terminate P2 receptor signaling, modulate receptor desensitization, alter specificities of the response or even generate signaling molecules (ADP) from precursors (ATP). (25) Extracellular ATP, UTP and UDP are also known to be potent growth factors for vascular smooth muscle cells through the activation of P2Y receptors. (9, 15) ATP, UDP, and others, acting at P2X7R and presumably also P2YR trigger IL-1 and TNF release from activated macrophages and endothelium. (8)

NTPDases are not only expressed on smooth muscle and endothelial cells. Dranoff et al. (9) have described expression of NTPDase 2/CD39L1 on portal fibroblasts where this specific localization may modulate novel functional interactions of these stromal cells.

Functions of CD39 may not be limited to vascular mechanisms such as platelet aggregation, (16) thromboregulatory functions (26) and other cell-cell interactions (18) crucial for the maintenance of the vascular integrity. Recently, infiltrating immune cells that dedifferentiate to ‘fibrocytes’ promoting scar formation have been described in lung, liver and pancreatic lesions. (4) Any proposed expression of CD39 by these cells and other vascular and stromal elements in scar tissues could play a significant role in the modulation of fibrogenesis within diseased pancreatic tissues and indeed elsewhere.

In this study we have explored patterns of expression of CD39 and P2-R in human normal pancreas, CP and PaCa. In addition, we have correlated the altered patterns clinical data with levels of expression of ectonucleotidases and P2-R with the clinicopathological state.
Materials and Methods:

Patients and Materials

Control pancreatic tissue samples were obtained from 9 organ donors apparently free of disease (4 women and 5 men). The median age of the organ donors was 41 years (range 35 to 61 years). All normal tissue samples were obtained from the organ donor's pancreatic head region. Chronic pancreatitis tissues were obtained from 11 patients suffering from alcoholic chronic pancreatitis (3 women and 8 men, median age 47, range 32 to 53 years) undergoing pancreatic head resection for refractory pain. All had severe morphological changes within the pancreas, such as duct dilation or duct occlusion with impacted pancreatic ductal stones. In this group with chronic pancreatitis, important clinicopathological characteristics such as diabetes, fibrosis and cachexia are listed in Table 1. Pancreatic cancer tissues were obtained from 28 patients (12 women and 16 men) undergoing partial duodenopancreatectomy (Whipple resection) for pancreatic cancer. The median age of the pancreatic cancer patients was 64 years (range 38 to 77 years). There were six stage I, two stage II, fifteen stage III and five stage IV tumors according to tumor-node-metastasis (TNM) classification and histopathological grading system of the International Union Against Cancer (Table. 2). (27) Tumor grading showed 5 well-differentiated tumors, 11 moderately differentiated tumors, and 12 poorly differentiated tumors (not shown).

In all experiments, tissue sections were simultaneously and equally processed to ensure uniformity of data. Freshly removed tissue samples were immediately snap frozen for immunohistochemistry. Concomitantly, tissues for RNA extraction were snap frozen in liquid nitrogen in the operating room upon surgical removal and maintained at −80°C until use.
The studies were approved by the Human Subject Committee of the University of Heidelberg, Heidelberg, Germany.

Real-Time RT-PCR detection

Tissue samples were collected in RNAlater (Ambion, Austin, TX, USA), and stored at –20°C until analysis. Tissue was disrupted by one run with the RiboLyser (ThermoHYBAID, Heidelberg) in lysing matrix “D” tubes (Q-BIOgen, Heidelberg) containing 400 µl lysis buffer from the MagnaPure mRNA Isolation Kit II (ROCHE Diagnostics, Mannheim). The RiboLyser tubes were centrifuged at 4°C for 1 min at 13000 rpm. Then, 300 µl of the lysate was collected and mixed with 600 µl capture buffer containing oligo-dT. After centrifugation at 13000 rpm for 5 min, 880 µl of this mix was transferred into a MagnaPure sample cartridge and mRNA was isolated with the MagnaPure-LC device using the mRNA-II standard protocol. The elution volume was set to 50µl. An aliquot of 8.2 µl RNA was reverse transcribed using AMV-RT and oligo- (dT) as primer (First Strand cDNA synthesis kit, Roche) according to the manufactures protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 µl and stored at –20°C until PCR analysis.

Specific primer sets for CD39, CD39L1, P2X7, P2Y1, P2Y2 and P2Y6 optimized for the LightCycler (RAS, Mannheim Germany) were developed and purchased from SEARCH-LC GmbH, Heidelberg. The PCR was performed with the LightCycler FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided in the parameter specific kits. To control for specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was observed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR-cycle number (CP) at which the detected
fluorescence intensity reaches a fixed value. This approach dramatically decreased variations due to handling errors over several logarithmic dilution steps.

To correct for differences in the content of total RNA, calculated copy numbers were normalized according to the average expression of two housekeeping genes, Cyclophilin B and HPRT. Values were thus given as input adjusted copy number per µl of cDNA.

**Cell lysate preparation and Western Blot**

Cell lysates were prepared using a homogenate buffer (containing 20 mM Tris, 100 mM NaCl, 0.1 mM PMSF, 10 µg/ml aprotinin, 1% NP-40). After sonication and centrifugation (12000 rpm for 15min at 4ºC), protein concentrations were measured with the Bradford method (Bio-Rad Lab, Hercules, CA, USA). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis techniques were employed to separate proteins (40 µg/lane) on 4 to 15% linear gradient gel, both under reducing and non-reducing (only CD39) conditions. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) by semi-dry electroblotting, and probed with either CD39 (Ancell), CD39L1 rabbit polyclonal (BZ3-4F), P2X7 (Alomone, C-20, sc-15210), P2Y2 (Alomone, L-20, sc-15200), or for control GAPDH (Amblion) primary antibodies. P2Y1 and P2Y6 antibodies from Santa Cruz Biotechnology Inc. were found to be non-specific on testing and were not used further. Bands were visualized using horseradish peroxidase-conjugated secondary antibodies Donkey-anti-Rabbit (CD39L1, P2X7, P2Y2), Goat-anti-Mouse (CD39, GAPDH), and Mouse-anti-Goat (P2Y1) (1:85000; Pierce, Rockeford, IL, USA). Equal gel loading was either confirmed using Ponceau red staining (not shown) of the blotted membrane or by stripping the blots and reprobing with GAPDH antibody.
To validate P2 receptor expression (P2X7, P2Y2), primary antibodies against P2X7 and P2Y2 were blocked with the specific antigen, provided by the manufacturer (Alomone labs). Internal controls show complete negative blots (not shown) that confirmed that signals detected were specific.

For the semi-quantitative estimates of protein concentration by image densitometric analysis, the IMAGE Station 2000MM from Kodak® was used. Kodak1D software was used to digitalize data, according to the manufacturer’s protocols.

**Immunohistochemistry**

Surgical specimens of normal pancreas, CP and PaCa were embedded in Triangle Biomedical Sciences (TBS) tissue freezing medium (American Master Tech Scientific, Lodi, CA) and immediately snap-frozen in isopentane, cooled on liquid nitrogen, and stored at –80ºC. Five-micrometer serial cryostat sections were fixed in ice-cold acetone (Sigma) for 10 minutes and rinsed in phosphate-buffered saline (PBS). IgG binding sites were blocked with appropriate control serum diluted 1:5 in solution 9 (PBS, pH 7.4, supplemented with 0.1% bovine serum albumin, 150 mM tranexamic acid, 20 µg/mL aprotinin (3-7 trypsin inhibitory units per milligram), 1.8 mM ethylenediaminetetraacetic acid, and 2 mM iodoacetic acid) further supplemented with 2% 3-omega fatty acid (Sigma) for 1 hour at room temperature. Sections were then incubated overnight with primary antibody for CD39, CD39L1, P2X7 and P2Y2 at 4ºC, then rinsed with PBS, and incubated with 3% H2O2 in methanol for 5 minutes to deplete endogenous peroxidase. After incubation with the appropriate biotinylated IgG for F(ab’)2 fragment of IgG for 30 minutes, staining was performed with the Vectastatin ABC elite kit (Vector Laboratories) with diaminobenzidine as the peroxidase substrate. Sections were counterstained with Mayer’s hematoxylin, dehydrated, cleared in xylene, and mounted in Permount. All
antibodies were diluted in solution 9, unless otherwise indicated. Optimal antibody concentrations were determined by serial dilution.

Statistical Analysis
Results are expressed as median and range and/or mean ± SD. For statistical analysis the Mann-Whitney test was used. Survival curves were computed by the Kaplan-Meier method as analyzed by the log-rank test. Significance was defined as p<0.05. For survival analysis the samples were segregated according to the fold increase in mRNA expression levels compared with normal controls in positive and negative tumors. The cut-off levels (number of mRNA copies) were set for CD39 at 410 and P2Y2 at 33.

Results:
Altered mRNA levels of NTPDases and P2-R
RT-PCR was performed to determine mean levels of mRNA expression of CD39, CD39L1, P2X7, P2Y1, P2Y2 and P2Y6. CD39, CD39L1, P2X7 and P2Y1 were expressed at low levels in NP. P2Y2 and P2Y6 mRNA levels in NP were near zero. In CP, expression levels of mRNA for CD39 (5.0-fold increment, p=0.0006) and P2X7 (3.0-fold increment, p=0.0024) were significantly and substantially increased. In contrast, CD39L1 (1.3-fold increase, p=0.0122), P2Y2 (2.1-fold increase, p=0.0098) and P2Y6 (2.3-fold increase, p=0.0185) were only moderately overexpressed in CP, as compared to NP (Fig. 1).

Both of the NTPDases tested were highly overexpressed in PaCa (CD39: 13.3-fold increase, p=0.0007; CD39L1: 3.7-fold increase, p=0.0126). P2Y-R mRNA levels increased in PaCa as compared to normal pancreas (P2Y2: 11.7-fold increase, p=0.0008; P2Y6: 4.4-fold increase, p=0.0011), whereas P2Y1 and P2X7 were unchanged (P2Y1: 0.8-fold increase, n.s.) and P2X7 only moderately but not significantly
overexpressed (1.8-fold increase, n.s.) (Fig. 1). Interestingly, both NTPDases (CD39 and CD39L1) as well as P2Y2 and P2Y6 were significantly overexpressed in CP as well as PaCa, whereas P2X7 was not upregulated in PaCa. P2X7 transcripts were significantly upregulated in CP when compared to PaCa (p=0.0247), whereas those of P2Y2 were significantly overexpressed in PaCa, as compared to CP (p=0.0209).

**Overexpression of CD39 and purinergic receptors**

Low levels of CD39, CD39L1, P2X7, P2Y2 and P2Y6 expression were noted in normal representative pancreatic biopsies, (Fig. 2A). CD39 and especially P2X7 were upregulated and highly expressed in CP. CD39L1 and P2Y2 were moderately expressed in CP where levels were comparable with findings in normal pancreas. PaCa samples exhibited heightened levels of CD39 and P2Y2, validating mRNA findings of overexpression. High levels of both intact CD39 (78kDa) and truncated proteolytic fragments (50kDa) were observed. P2X7, expressed as a double band at about 79kDa, was overexpressed in CP. In contrast to elevated P2X7 in CP, P2Y2 (~47kDa) was only specifically overexpressed in PaCa (Fig. 2A). Measurements of protein expression in relation to GAPDH expression revealed a trend towards increased expression by 2.75-fold (p=n.s.) in the instance of CD39 levels in CP. In PaCa, CD39 expression was however, significantly increased by 2.75-fold (p=0.0070) (Fig. 2B). Expression levels of P2X7 show a 3-fold increase in CP and a 2.2-fold increase of protein expression in PaCa, when compared to P2X7 protein expression (in NP). Although at the mRNA level, P2X7 was overexpressed in CP when compared to NP, at the protein level, differences in P2X7 expression between CP and NP, did not achieve statistical significance (p=0.11). P2Y2 protein expression level appeared comparable in both NP and CP (1.7 time increase, p=n.s.) but there was a 3.1-fold increase in PaCa, as compared to NP, (p=0.0281).
Specific localization of CD39 and P2-R

Normal pancreas tissues revealed CD39 immunostaining within the vasculature, especially endothelial cells. Minimal or no staining of pancreatic ductal-, islet- or acini cells was observed. CD39L1 immunostaining was present in nerves and adventitial tissues around islet cells. Reactivity was also detected in interlobular fibroblast like cells, suggestive of pancreatic stellate cells. (2) P2X7 receptors were primarily expressed in macrophages and some other immune cells of normal pancreas, but not in parenchymal cells. P2Y2 showed faint to moderate immunostaining in parenchymal cells together with strong additional expression in adventitial layer of vessels (Fig. 3A, 3D, 3G, 3K).

In CP, NTPDases were greatly overexpressed. CD39 was localized to stromal tissue and vasculature of CP. CD39L1 was moderately expressed in fibroblast like structures. P2X7 in CP was localized to immune cells. P2Y2 expression levels were high in fibroblastic areas but not in pancreatic parenchyma (Fig. 3B, 3E 3H, 3L).

In PaCa, CD39 was highly expressed around malignant cells in stromal elements. However, cancer cells as well as normal acinar cells do not express CD39 to any extent. CD39L1 localization to nerves can be clearly shown in Figure 3F, where strong CD39L1 signals are detected. Interseptal fibroblasts also stain moderately for CD39L1. Moderate to weak signals are encountered in the cytosol of cancerous ductal cells and acini. P2X7 was almost undetectable in pancreatic cancer, other than clear positive staining in immunocytes. P2Y2 expression retained specific expression in interseptal fibroblasts but not ductal and acini cells. The signal intensity is high and consistent with Western Blot and mRNA data. P2Y2 faintly expressed in the common fibroblast like cells of the abundant present desmoplastic reaction (Fig. 3C, 3F, 3I, 3M).
High CD39 expression in PaCa correlates with better clinical outcomes

To evaluate the clinical significance of mRNA expression in pancreatic cancer samples, RT-PCR results were correlated with survival data and histopathological parameters after tumor resection (Table. 2). Patients where tumors exhibited overall elevated high levels of CD39 mRNA, had significantly longer median postoperative survival periods (CD39: median 33 months; mean ± SD 29.7 ± 20.94 months) than patients whose tumors exhibited low to moderate CD39 mRNA levels (CD39: median 14 months; mean ± SD 20.1 ± 16.56 months). These differences were statistically significant when analyzed by the log-rank test (CD39: p=0.0466) (Fig. 4). There were no significant relationships noted for CD39L1, P2Y2 and P2Y6 mRNA expression, postoperative patient survival and tumor stage (Table. 2).

Clinicopathological features in patients with CP (Table. 1) did not show correlations between expression levels of ectonucleotidases or P2-R and the intensity or severity of fibrosis. No clinical parameters, such as diabetes or cachexia, could be correlated with specific patterns of expression of CD39 ectonucleotidases or of P2-R.
Discussion:

In normal pancreatic tissues, NTPDases are localized to vascular structure and nerves (especially CD39L1). P2X7 are expressed at low levels by the few macrophages and immunocytes in normal pancreas but not by the parenchymal cells. P2Y2 is noted in adventitial tissues of the pancreatic vasculature and at low levels in parenchymal cells. Within areas of chronic pancreatic inflammation and fibrosis, the tissue distribution/expression of NTPDases as well as P2-R dramatically change. Transforming fibroblasts express increased levels of CD39, P2Y2 and P2Y6, whereas infiltrating leukocytes show high expression of P2X7-receptor. In pancreatic cancer CD39, CD39L1, P2Y2 and P2Y6 are all upregulated. CD39 is highly expressed around malignant cells and not localized to acini and ductal cells. In contrast P2Y2 expression retained specific expression in interseptal fibroblasts. P2X7 is mainly expressed on mononuclear cells, such as macrophages, as shown in a previous study (17). Our data clearly show overexpression of ectonucleotidases and select P2-receptors in human CP and PaCa.

The data obtained from control biopsies might explain observed differences in the sensitivity of normal pancreas to purinergic agonists. Interestingly, acinar cells do not express P2X7 receptors, whereas pancreatic ductal cells do. (24, 28). In general, pancreatic ducts show little desensitization of purinergic receptors, but only very few pancreatic acini show functional P2 receptors. (24, 28) Pancreatic acini release ATP after cholinergic stimulation and this may lead to selective signaling, as acini lacking P2-expression could avoid being stimulated by the released ATP.

Inflammation in pancreatic disease is a complex pathophysiological process associated with leukocyte infiltration, tissue injury, cell death, release of nucleotides and cytokines. These actions are relevant stimuli for altered NTPDase activity and increased levels of
hydrolysis of ATP and ADP that could modify P2-R mediated responses under inflammatory conditions. For example, P2X7 receptor activation of caspase-1 by macrophages is dependent upon prestimulation of these cells with LPS or other Toll-like receptors (TLR) ligands (17). In addition, P2Y receptors induce downstream effects via inositol triphosphate (IP3)-mediated pathways that are also modulated by several other signals (10).

Possible links between fibrosis and the impact of P2-R in modulating hepatic fibrogenesis have been proposed by Dranoff et al. (11) Hepatic stellate cells (HSC) expressing P2Y subtypes differ in their relative activation states, with high expression of P2Y2 and P2Y4 under basal or quiescent states, whereas HSC, activated by ATP and UTP, express high levels of P2Y6. Activation of HSC also leads to heightened expression of CD39L1/NTPDase2. Linked to this mechanism, extracellular UDP can be shown to triple mRNA levels of procollagen-1, a major constituent of extracellular matrix. (11) P2Y receptors (P2Y2 and P2Y6) are highly expressed in fibroblast-like cells and may regulate procollagen-1 transcription in pancreatic disease. These data indicate interesting future targets for pharmacological interventions in fibrogenic diseases.

Comparable factors might be associated with the observed upregulation of NTPDases and P2Y receptors in pancreatic cancer. Furthermore, our data suggest (at least in part) that there are correlations between possible CD39 and P2-R interactions/regulation (e.g. P2Y1 and P2Y2) within tumor matrix in human pancreatic cancer.

Further considerations of the impact of NTPDases on fibrosis and specific localizations have been described. (9) CD39L1 expression has been shown on the plasma membrane of fibroblast-like cells adjacent to the basolateral aspect of bile duct epithelia (the area where portal fibroblast are located). (9) Our findings show similar patterns for pancreatic fibroblast like cells around pancreatic ducts in normal pancreas. In PaCa, but not CP, CD39L1 expression is highly upregulated. These alterations in NTPDase
expression might alter extracellular nucleotide signaling in pancreatic periductular fibroblasts, in the setting of malignancy and desmoplasia.

Recent studies in mouse pancreas show that CD39 is mainly expressed in endothelium but not in ductal cells. (20) By electron microscopy, granules in mouse pancreatic acini are shown to express CD39 and CD39L1. Additionally Sorensen et al. (28) have described ATP as a potential paracrine regulator between acini and ducts. Furthermore, CD39 can be released with a suitable stimulus into the lumen. (28) Differences in CD39 localization and distribution of P2 and adenosine receptors might be responsible for functional alterations in the acinar-ductal system of injured rat pancreas.

In comparison to the upregulation of CD39 in desmoplastic area of PaCa, we have demonstrated upregulation of CD39 expression in CP, a novel finding not yet described, Kittel et al. (19) have demonstrated CD39 expression, albeit low, in cancerous ducts with only weak staining of connective tissues around the duct. We also show significant correlations between expression of CD39 and postoperative survival of patients with PaCa. These data might infer that samples derived from patients with low CD39 expression are linked to later stage malignancy. This would support our hypothesis that high CD39 expression in the tumor matrix might provide “protective” effects for patients with PaCa.

These findings potentially implicate distinct roles of CD39 and purinergic signaling in tissue remodeling, fibrogenesis and probably tumor progression. P2-receptors might become important mediators of downstream effects of nucleotide processing and also show modulation in inflammatory and neoplastic conditions. Mechanisms of down-regulation of CD39 expression during tumor progression are not completely understood. It is possible that induction of CD39 is an initial trigger of P2-R upregulation/desensitization in inflammation and desmoplasia. The specific localization of CD39 in pathological pancreas tissues, mainly on fibroblasts and endothelium as well
as P2Y2 specific on fibroblasts, might link pancreatic inflammation and desmoplasia to purinergic signaling.

P2X7 is known to have pro-inflammatory properties and is connected to cytokine release (e.g. IL-1β, IL-18), NO generation and cytotoxicity and other relevant processes in inflammation. (3, 7, 14) Their various P2X and P2Y receptors are expressed in immunocytes. P2X7 has been demonstrated to be a functional receptor in the majority of these immune cells. (6) We have shown that P2X7 is highly upregulated in CP but not in pancreatic cancer and might be therefore linked with pancreatic inflammation. P2X7 may serve as an immunomodulator in this disease via modulated actions of tissue macrophages, other immune and inflammatory cells (Fig. 3H).

Expression of P2X7 receptors in diseased pancreatic acini, may however be pathogenetic, as acinar cells are packed with nucleotide granules, which might be activated and cause tissue injury. The ATP release from acini may initiate its action downstream of the acini level, in ductules and larger pancreatic ducts. (28)

The fact that P2Y2 is highly upregulated at the mRNA level in PaCa and the localization in fibroblasts in close vicinity to invading cancerous ducts may contribute to distinct roles in cancer progression/inhibition by facilitating angiogenesis or fibrogenesis. These processes of tissue remodeling are known to be highly dysregulated in pancreatic cancer. Acini contain low numbers of functional P2 receptors. (24) This may indicate that changes in the pancreatic tissue P2-repertoire of receptors results in disturbed purinergic signaling.

Progressive tissue changes result in pancreatic parenchymal atrophy, fibrosis and longterm pancreatic insufficiency. The roles of the inflammatory actions as well as the desmoplastic process in chronic pancreatitis and pancreatic cancer are still poorly understood. However, this present study provides insights into possible mechanistic involvement of purinergic signaling and NTPDases in diseases of the pancreas.
Overexpression of CD39 and correlation to longterm survival after tumor resection for pancreatic cancer also suggest potential, highly novel role for purinergic signaling in PaCa.

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GRANTS

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Table and Figure legend:

**Table. 1.** Clinicopathologic features of 11 patients with chronic pancreatitis. Diabetes, severity of fibrosis and the presence of cachexia are detailed.

**Table. 2.** Clinicopathologic features of 28 patients with pancreatic cancer are listed. TNM classification has been established according to UICC guidelines.

**Fig. 1.** Relative mRNA expression for normal pancreas, chronic pancreatitis and pancreatic cancer is shown for CD39, CD39L1, P2X7, P2Y1, P2Y2 and P2Y6. Expression has been standardized to Cyclophilin B and HPRT and is shown as number of copies of the appropriate gene.

**Fig. 2. A.** Assays of CD39, CD39L1, P2X7 and P2Y2 protein expression by Western Blot analysis. Representative protein samples from whole tissue lysates of normal pancreas, chronic pancreatitis and pancreatic cancer were electrophorized, blotted and consecutively incubated with specific primary antibodies. CD39, CD39L1 and P2Y2 showed clear increase of expression in PaCa. Internal control is GAPDH.

**B.** Protein concentrations were determined by densitometric measurements of Western blots. Samples were standardized to GAPDH for internal control and expressed as arbitrary units. Statistical analyses revealed high level CD39 protein expression in PaCa (**p=0.0070** with a trend towards significance in CP (**p=0.0571**), when compared to NP. No statistical correlations existed for P2X7 when analyzing whole tissue extracts. For P2Y2 protein expression there was significant overexpression in PaCa (**p=0.0281** but not for CP, when compared to NP.
**Fig. 3.** Immunostaining of CD39 (A-C), CD39L1 (D-F), P2X7 (G-I) and P2Y2 (K-M) in normal pancreas (A, D, G, K), chronic pancreatitis (B, E, H, L) and pancreatic cancer (C, F, I, M). Original magnification x200. High expression of CD39 in fibroblasts of CP and participation in the desmoplastic reaction in PaCa is shown. CD39L1 is expressed by nerve fibers (3D and 3F). P2X7 is highly expressed in inflammatory cells in CP (3H). P2Y2 protein expression is high in fibroblasts close to tumor cells in PaCa (3M).

**Fig. 4.** Survival curves. Kaplan-Meier plots of the postoperative survival periods in patients whose tumors exhibited concomitant overexpression of CD39 (A) (cutoff ≥ 410) and P2Y2 (B) (cutoff ≥ 33) vs. patients whose tumors exhibited only low number of mRNA copies of CD39 (A) (cutoff < 410) and P2Y2 (B) (cutoff < 33). Log-rank analysis indicated (p=0.0466) a significant difference in survival periods for high expression of CD39.
References:


Fig. 1.

![Graph showing relative mRNA expression of CD39, CD39L1, P2X7, P2Y1, P2Y2, and P2Y6 in normal pancreas, chronic pancreatitis, and pancreatic cancer.]

- **CD39**: Normal Pancreas = 0.591
  - Chronic Pancreatitis: *p=0.0591
  - Pancreatic Cancer: *p=0.0024

- **CD39L1**: Normal Pancreas = 0.0247
  - Chronic Pancreatitis: *p=0.0209

- **P2X7**: Normal Pancreas = 0.0024
  - Chronic Pancreatitis: *p=0.0024
  - Pancreatic Cancer: *p=0.0209
Fig. 2.

A

kDa

CD39

CD39L1

P2X7

P2Y2

GAPDH

B

CD39

**p=0.0070

P2X7

*p=0.0281

P2Y2
Fig. 3.

CD39

A  Normal Pancreas  B  CP  C  PaCa

CD39L1

D  E  F

P2X7

G  H  I

P2Y2

K  L  M
Fig. 4.

A

Low CD39
High CD39 *

*p=0.0466

B

Low P2Y2
High P2Y2

p=0.1790
Table. 1. Clinicopathologic features of patients with CP

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<th>Age [yrs]</th>
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Table. 1. Clinicopathologic features of 11 patients with chronic pancreatitis. Diabetes, severity of fibrosis and the presence of cachexia are detailed. Cachexia: more than 10% loss of body weight in the past 6 months before admission.
Table 2. Clinicopathologic features of patients with PaCa

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Table 2. Clinicopathologic features of 28 patients with pancreatic cancer are listed. TNM classification has been established according to UICC guidelines.