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Disordered Pancreatic Inflammatory Responses and Inhibition of Fibrosis in CD39-null mice

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Abstract

Background & Aims—Extracellular nucleotides are released from injured cells and bind to purinergic-type 2 receptors (P2-R) that modulate inflammatory responses. Ectonucleotidases, such as CD39/NTPDase1, hydrolyze extracellular nucleotides to integrate purinergic signaling responses. As the role of extracellular nucleotides and CD39 in mediating inflammation and fibrosis are poorly understood, we studied the impact of CD39 gene deletion in a model of pancreatic disease.

Methods—Pancreatitis was induced by cyclosporine pretreatment, followed by cerulein injections (50 µg/kg, 6 i.p. injections daily, 3 times per week); mice were sacrificed at days 2, wk 3 and wk 6. Experimental parameters were correlated with cytokine levels in blood, mRNA and protein expression of purinergic and fibrosis markers in tissues. Immunohistochemistry and pancreatic morphometry of fibrosis were performed in wild-type and CD39-null mice. Effects of CD39 deletion on proliferation of primary pancreatic stellate cells (PSC) were investigated *in vitro*.

Results—Wild-type mice developed morphological features of pancreatitis with the anticipated development of parenchymal atrophy and fibrosis. CD39 and P2-R became overexpressed in vascular and adventitious wild type tissues. In contrast, CD39-null mice had inflammatory reactions but developed only minor pancreatic atrophy and limited fibrosis. Interferon- γ became significantly increased in tissues and plasma of CD39-null mice. Wild-type PSC expressed high levels of CD39 and P2-R. CD39-null PSC exhibited decreased rates of proliferation and the expression of procollagen- α 1 was significantly inhibited *in vitro* ($P < 0.03$).

Conclusions—CD39 deletion decreases fibrogenesis in experimental pancreatitis. Our data implicate extracellular nucleotides as modulators of PSC proliferation and collagen production in pancreatitis.

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Introduction

Chronic pancreatitis (CP) is an important disease that results in organ failure and fibrosis. CP is characterized pathologically by inflammatory infiltrates, progressive organ atrophy and fibrosis.¹ Fibrogenesis in pancreatitis develops as the result of acinar cell injury and necrosis that is in turn followed by cascades of inflammation and fibrosis. Activation of pancreatic stellate cells (PSC), with stimulated synthesis of extracellular matrix (ECM) and reduced matrix degradation, results in overall matrix accumulation.^{2, 3} PSC are located in interlobular areas and in interacinar spaces and share many phenotypic features with hepatic stellate cells (HSC) e.g. expression of α -smooth muscle actin (α -SMA) (>90% of cells)⁴ and express desmin (20–40%).⁴ Transforming growth factor- β (TGF- β) and platelet derived growth factor (PDGF), both serve as profibrogenic mediators stimulating ECM synthesis.⁴

Inflammation and repetitive pancreatic injury result in cytokine-mediated activation and oxidant damage to cells⁵ with potential for increased extracellular nucleotide release and accumulation. Extracellular nucleotides are hydrolyzed by ecto-enzymes such as nucleoside triphosphate diphosphohydrolase-1 (CD39/NTPDase-1).⁶ CD39 is the dominant vascular endothelial and immune ectonucleotidase and hydrolyzes both ATP and ADP in the plasma to AMP, ultimately to adenosine via CD73.⁶ Another NTPDase associated with the vasculature and expressed by HSC and portal fibroblasts is the cell-associated ecto-ATPase (CD39L1 or NTPDase2).⁷ Extracellular nucleotides regulate inflammation and immunity via purinergic/pyrimidinergic P2-receptors (P2X-R and -P2Y-R receptors).^{8, 9} NTPDases functionally interact with these P2Y-G-protein coupled receptors.⁶ Importantly, combinations of NTPDases have the capacity to terminate P2 receptor (P2-R) signaling, modulate receptor desensitization, alter specificities of the response or even generate signaling molecules (ADP) from precursors (ATP).¹⁰

Extracellular ATP, UTP and UDP are known as potent growth factors for vascular smooth muscle cells and act via P2Y receptors.^{7, 11} ATP, UDP, and others, acting at P2X7R and presumably also other P2YR trigger IL-1 and TNF release from activated macrophages and endothelium.¹²

Functions of CD39 may not be limited to regulation of vascular mechanisms such as platelet aggregation,¹³ thrombosis¹⁴ and maintenance of the vascular integrity.¹⁵ Recently, we reported CD39 and certain P2-R overexpressed in human pancreatic cancer and CP.¹⁶ CD39 is localized to vasculature and stromal elements, such as PSC in normal and diseased pancreas. Altered expression patterns of CD39 are seen in CP and pancreatic cancer. We also noted P2X7 to be upregulated in CP and being localized to immune cells.¹⁶

We next addressed whether purinergic signaling and in particular CD39 could impact pancreatic fibrosis. To test this hypothesis, we examined the course of pancreatic fibrosis and inflammation in CD39-null mice. We applied an established model of cerulein-induced pancreatitis that causes necro-inflammatory injury by hyperstimulation of pancreatic acinar cells.¹⁷

In this study, we charted the course of pancreatitis from acute (day 2) to late stages of pancreatic injury (wk 6), as per standard methodology,^{18–20} by examining expression of CD39, CD39L1, P2-R, fibrogenic factors and cytokines *in vivo*. Morphological and systemic parameters were determined, and fibrosis scores were applied. We next determined primary PSC proliferation properties and show substantial phenotypic differences *in vitro* between wt and CD39-null PSC that underpin the differences seen *in vivo*.

Materials and Methods

Animals

Pathogen-free wild-type and CD39-null C57BL6 mice, each weighing 25–35 g, were used in accordance with standard Institutional Animal Welfare guidelines, Beth Israel Deaconess Medical Center, Harvard University, Boston, USA. CD39-null mice were derived, as described elsewhere.⁶ C57BL6 CD39-null mice were back-crossed at least six times and genotyping of the CD39 alleles was performed, as previously described.⁶ Wild-type C57BL6 mice were purchased from Charles River laboratories® (Wilmington, MA, USA).

Experimental pancreatitis and tissue processing

Cerulein induced pancreatitis (CIP) in wt (n=19) and CD39-null (n=16) was induced by administration of 6 intraperitoneal injections of 50 µg/kg body weight cerulein (Sigma-Aldrich, St. Louis, MO, USA) per day, 3 times a week every alternative day for up to 6 wks. Control wild-type and mutant animals received a comparable volume of 0.9% sodium chloride (saline). Cyclosporine A (cyclosporine injection, USP, Bedford Labs™, Bedford, OH, USA) was injected intraperitoneally²⁰ at a dose of 10 mg/kg body weight once a day for 7 consecutive days prior to the cerulein administration, as previously described.²⁰ Mice were examined daily for signs of diarrhoea or symptoms of distress (lethargy, periorbital exudates and piloerection). Once weekly, body weight was measured and blood sugars checked.

Mice were sacrificed at indicated time points (day 2, wk 3 and wk 6) as per standard practice^{18, 19} by overdose of pentobarbital. Pancreatic tissues were removed, weighed and embedded in TBS-Tissue freezing medium™ (American Mastertech, Lodi, CA, USA) and snap frozen in isopentane, cooled on liquid nitrogen and stored at –80°C for immunohistochemistry. Tissues for RNA extraction and protein analyses were snap frozen in liquid nitrogen and maintained at –80°C until use.

Determination of IFN-γ levels in blood serum

Levels of IFN-γ in serum, isolated from wt and CD39-null mice at 3 and 6 wks after induction of cerulein pancreatitis, were determined using the MIF00-ELISA-kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

Amylase analysis

Amylase levels in blood plasma were determined using the AMY2–125 amylase liquid kit from Global Medical Instrumentation Inc. (GMI, Ramsey, MN, USA), according to manufacturer's protocol (sample dilution 1:2).

Expression of CD39, purinergic receptors and matrix parameters

RNA(RT-PCR) studies—Extracted mRNA from mouse pancreas was DNase treated and reverse transcribed using the SuperScript kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations using random hexamer primers for CD39, CD39L1, P2X7, P2Y2, CD31, CD34, CD45, α-SMA, procollagen-α1, TGF-β, IFN-γ, MMP-2, MMP-9, MMP-13 and TIMP-1 (Applied-Biosystems, Foster City, CA, USA). Quantitative PCR (Q-PCR) was performed using the 7700 Sequence Detector (Applied-Biosystems) and TaqMan technology as previously described.¹⁶ Data were analyzed using the relative standard curve method, and expression levels were normalized to the expression of 18S ribosomal subunit as internal controls.

Western Blots—Cell lysates were prepared and (SDS)-polyacrylamide gel electrophoresis techniques employed to separate proteins exactly as previously described.¹⁶ The membranes

were probed with either CD39 (C9F), CD39L1 (BZ3–4F), P2X7 and P2Y2 (Alomone Labs Ltd., Jerusalem, Israel), MMP-2 (Chemicon Int., Temecula, CA, USA), TGF- β 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), CD45 (Chemicon), fibronectin and α -SMA (Sigma, St. Louis, MI, USA) or for control anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Amblion Inc., Woodward, TX, USA) primary antibodies. Appropriate secondary antibodies were used for detection, as previously described.¹⁶ Equal gel loading was confirmed by analyses with GAPDH antibody.

To further 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 signified signals detected were specific.

Histological Analysis and Grading of Pancreatic Lesions

Masson's trichrome and Hematoxylin-Eosin (H&E) stainings were performed. Five-micrometer pancreatic sections were evaluated for parenchyma structure, fibrosis, inflammation, atrophy and necrosis. Two qualified observers independently analyzed the non-overlapping histological sections in a blinded manner. Severity of cerulein induced pancreatitis was graded by a semi-quantitative scoring system as previously described.¹⁹ Briefly, pancreatic sections, were investigated regarding parameters such as tissue architecture changes (graded as: 0=absent, 1=rare, 2=minimal (<10%), 3=moderate (<50%) and 4=major (>50%) of the total parenchyma affected), fibrosis (0 = absent, 2=only within areas, and 4=diffuse), glandular atrophy (0=absent, 1=minimal, 2=moderate, 3=major) and presence of pseudotubular complexes (0=absent, 1=minimal, 2=moderate, 3=major). These parameters were graded and a total score was calculated for each pancreatic specimen.

Immunohistochemistry and immunofluorescence microscopy

Immunohistochemistry for CD39 (C9F), CD39L1 (BZ3–4F), P2X7 (Alomone Labs, Jerusalem, Israel), and CD34 (Santa Cruz Biotechnology) was performed exactly as previously described.¹⁶

Tissue sections for colocalization studies of CD39 and CD34 were fixed in 2% paraformaldehyde. Sections were incubated with primary antibodies (CD39, CD39L1 and CD34) at 4 °C over night. After incubation with secondary antibodies (Fluorescein isothiocyanate (FITC)-labeled anti-rabbit secondary IgG for CD39 and cytochrome 3 (Cy3)-conjugated anti-rat IgG to detect CD34, (Molecular Probe, Eugene, OR, USA)) were applied.

Immunofluorescence microscopy for PSC *in vitro* was performed for α -SMA and desmin as described.²¹ Cells were seeded in DMEM (Sigma Aldrich) (containing L-Glutamine with 10% fetal calf serum, penicillin, streptomycin and amphotericin B) on 1 cm² glass cover slips (Lab-Tek Chamber Slide™, Nalge Nunc, Rochester, NY, USA).

For nuclear counterstaining, Hoechst 33258 (pentahydrate (bis-benzimide), Molecular Probes, 1:10000) was used.

PSC culture and identification

PSC from wt and CD39-null mice were isolated by outgrowth methods using pancreatic tissue blocks according to the technique described for human PSC by Bachem et al.⁴ In brief, small 1–1.5mm³ sized tissue blocks were seeded in 6-well plates in the presence of DMEM (Sigma Aldrich, St. Louis, MO, USA) containing standard cell culture medium and supplements as described above. Twenty-four hours after seeding, culture media was changed, and on day three tissue blocks removed. Confluent cells were subcultured by trypsinization using 0.05%

trypsin solution containing 0.02% ethylenediaminetetraacetic acid (EDTA). PSC were identified by immunocytochemical staining for both α -SMA and desmin.

PSC proliferation in response to PDGF and TGF- β

PSC were exposed to increasing concentrations of the PDGF isoform PDGF-BB (5 ng/ml) (ProSpecTechnoGene, Rehovot, Israel) and TGF- β 1 (2 ng/ml) (R&D Systems, Inc., Minneapolis, MN, USA) together with medium containing different concentrations of exogenous ATP (10, 50, 150 and 400 μ mol ATP). Cells incubated in medium without PDGF-BB or TGF- β 1 were used as controls. At least three independent experiments were performed for each instance.

The rate of cell proliferation after exposure to PDGF-BB and TGF- β 1 was assessed by initial cell counts per well augmented by determination of rate of DNA synthesis, as measured by the incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA of actively proliferating cells. We used the QIA58 BrdU Cell Proliferation Assay from Calbiochem according to manufacturer's protocol (Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany).

Statistical analysis

Data in this study are presented as means $\pm$ standard deviation (SD) of values (obtained from at least 4 mice per group and/or at least 3 independent *in vitro* experiments). All histology, Western blots and immunohistochemical images are representative of at least 4 mice per group. Statistical analysis of data was performed using the GraphPad PRISM4 software (GraphPad Software Inc., San Diego, CA, USA). Mean values of the experimental groups were compared using nonparametric testing (Mann-Whitney U test) for pair wise comparison and 1-way or 2-way ANOVA analysis for multiple comparisons. Values of $P < 0.05$ are considered statistically significant.

Results

Experimental outcomes

In this present study, we studied wild-type (wt) and CD39-null C57BL6 mice at 12 to 16 wks old. Mice in both groups had comparable body weights (wt 30.85 ± 2.87 g vs. CD39-null 33.14 ± 2.95 g; n.s.). Sham-treated CD39-null animals (saline injection i.p. for 6wks) also revealed no differences between pancreatic weights and morphology (Figures 1B, 3A), when compared to matched wt controls at all time points. In the CIP groups, pancreas weights were significantly decreased at 6 wks in wt (0.101 ± 0.017 g), when compared to those obtained from CD39-null mice (0.152 ± 0.028 g) ($P < 0.005$, Figure 1A). Mortality was not significantly different between wt (15%) and CD39-null (0%) mice. Serum amylase was measured to define the enzymatic inflammatory response in pancreatitis and confirmed comparable levels of elevation with CIP (Figure 1C) or sham treated animals (Figure 1D). At wk 3, CD39-null mice had significant higher serum levels of IFN- γ when compared to wt mice ($P < 0.05$) (Figure 1E).²² Blood sugar levels remained normal in all treatment groups and were not significantly different (Figure 1F).

Altered NTPDases and P2-R expression in cerulein induced pancreatitis (CIP)

RT-PCR was performed to determine levels of mRNA expression of purinergic elements (CD39, CD39L1, P2X7, P2Y2), angiogenic endothelial markers (CD31); genes as related to fibrogenic processes (TGF- β , α -SMA, procollagen- α 1, CD34, CD45) and angiostatic factors such as metalloproteinases (MMP-2, MMP-9 and MMP-13), TIMP-1 together with defined cytokines (IFN- γ) (Table 1).

The baseline mRNA expression levels in quiescent pancreas for all these parameters, except obviously for CD39, did not significantly differ between wt and CD39-null mice (Table 1A).

However, after treatment with cerulein at 3 wks, mRNA levels were altered in line with heightened inflammation and significant differences were observed for CD39, CD39L1, P2Y2, IFN- γ and TIMP-1 mRNA expression at 3 wks of CIP (Figure 1B).

Furthermore, procollagen- α 1 and α -SMA mRNA expression was upregulated in pancreatitis at 3 and 6 wks in wt and CD39-null mice but were not significantly different between wt and CD39-null mice (not shown).

After 6 wks of CIP, CD39, MMP-2 and MMP-9 were significantly upregulated in wt mice, when compared to CD39-null mice ($P < 0.05$) (Table 1C).

CD39 and P2X7 are overexpressed in CIP

CD39 protein levels increased peaking at wk 6 in wt tissues (Figure 2A). CD39L1 protein levels were markedly overexpressed in CD39-null mice at the 3 wk point but expression was equivalent to wt at 6 wks. P2X7 expression preferentially increased in CD39-null pancreas lysates at 3 wks (Figure 2A).

P2Y2 expression in CD39-null pancreas was increased compared to baseline at 6 wks. MMP-2 protein expression was significantly increased in CD39-null mice at 3 and 6 wks time, however only minor expression was noted in wt mice. TGF- β 1 protein expression peaked at wk 3.

More pronounced CD45 expression was detected in CD39-null than wt pancreatic lysates at 3 wks, albeit comparable expression was noted at 6 wks (Figure 2A).

Fibronectin was highly expressed during pancreatitis in wt and CD39-null mice at 3 and 6 wks, with substantive increased expression in wt pancreatitis noted at wk 6 (Figure 2A). α -SMA expression was induced at 3 and 6 wks of pancreatitis but these changes were not significantly different in wt tissues when compared to CD39-null pancreatic lysates (Figure 2A).

In pancreatic stellate cells (PSC) (Figure 2B), CD39 was detectable at high levels in wild-type PSC only. P2X7 was also highly expressed in wt PSC but only faintly noted in CD39-null PSC at the proteolytic cleavage fragment at ~ 60 kDa. The expression of CD39 on PSC *in vitro* is consistent with findings in immunohistochemical pancreatic tissue staining, whereas PSC are positive for CD39 (insert Figure 4C). Furthermore, wt PSC were shown to have associated higher levels of fibronectin and fragments when compared to CD39-null PSC (Figure 2B).

CD39 impacts evolution of fibrosis in CIP

Typical injury to pancreas can be characterized by distinct differences in parenchymal tissue changes indicated by: more intense fibrosis (Masson's Trichrome staining, Figure 3B), more severe tissue atrophy and disruption of tissue architecture observed in wt mice when compared to CD39-null mice. The different histological scores for fibrosis ($P < 0.02$) (Figure 3C), atrophy ($P < 0.02$) (Figure 3D), indicated significant parenchymal tissue protection in CD39-null mice when contrasted to wt.

Immunohistochemistry of chronic pancreatitis and PSC *in vivo*

In normal pancreas and cerulein induced pancreatitis, CD39 can be localized to endothelial cells and stromal compartments. This suggested positive staining for PSC and other pancreatic fibroblasts. There was minimal CD39 reactivity for pancreatic parenchymal cells (Figure 4A, C). Control slides for CD39-null mice revealed complete absence of immunoreactive CD39 staining (Figure 4B, D). P2X7 reactivity was localized to inflammatory cells (mostly

monocytes or macrophages) in CP at 6 wks in wt and CD39-null tissues (insert Figure 4E, F) but also showed reactivity on fibroblast like structures in periacinar spaces, where PSC were typically present (Figure 4E, F).

CD34 was generally detected in structures around pancreatic acini and ductal cells, where PSC, perivascular fibroblasts and other tissue fibroblasts are located. (Figure 5A, B).

Double staining of CD39 and CD34 and immunofluorescence microscopy revealed overlapping expression indicating staining of pancreatic fibroblasts *in vivo* (insert Figure 5C). Furthermore, CD39L1, known to be expressed and localized to portal fibroblasts,⁷ also colocalized with CD34 (Figure 5E, F).

Pancreatic stellate cells from CD39-null mice exhibit impaired proliferation rates

The growth and proliferation rates of CD39-null PSC under basal conditions and after stimulation with PDGF of CD39-null PSC were significantly lower, as compared to wt PSC ($P < 0.02$, Figure 6A). Furthermore, PSC from CD39-null mice were non-responsive to TGF- β (Figure 6A).

At increased levels of ATP (50 μ mol of ATP), proliferation rates of wt and CD39-null PSC were decreased after stimulation with PDGF and TGF- β (Figure 6B), when compared to basal conditions. Increased levels of ATP (basal, 10, 50, 150 and 400 μ mol ATP) in cultures of PSC stimulated proliferation in wt PSC (Figure 6C), whereas CD39-null PSC showed no major effect/response to increased levels of ATP. Only 10 and 50 μ mol ATP indicated beneficial significant proliferative effects on wt but not CD39-null PSC ($P < 0.05$) (Figure 6C). Phenotypic differences in PSC were noted for CD39 and P2X7 only (Figure 6D), whereas direct staining of CD39-null PSC for α -SMA *in vitro* revealed no differences. Comparable levels of α -SMA on mRNA and protein level indicate comparable rates of differentiation and viability of wt and CD39-null PSC (Figure 6E, F).

CD39-null PSC have altered expression of P2-R and show decreased procollagen- $\alpha 1$ expression

Under basal cell culture conditions, CD39 was expressed on wt PSC with minimal changes in levels of expression following stimulation with either PDGF or TGF- $\beta 1$ (Figure 7A). CD39L1 became overexpressed on CD39-null PSC during stimulation with PDGF ($P < 0.01$) (Figure 7B). Primary untreated wt PSC, revealed significantly increased P2X7 mRNA expression when compared to CD39-null mice ($P < 0.03$). This effect however disappeared during stimulation with PDGF and TGF- $\beta 1$ (Figure 7C). P2Y2 was only at levels of detection (Figure 7D).

Differentiation, indicated by α -SMA, was comparable between wt and CD39-null PSC (Figure 7E). Collagen production, as measured by procollagen- $\alpha 1$ mRNA was significantly increased in wt mice ($P < 0.03$), and could be induced under TGF- $\beta 1$ stimulation in wt PSC but not in CD39-null PSC. No synergistic boosting effect of PDGF could be detected in CD39-null PSC (Figure 7F).

Discussion

Purinergic signaling is known to play an important role in thromboregulatory disorders, vascular disease, transplantation rejection and processes of hepatobiliary fibrosis.^{7, 23-26} CD39 is the dominant vascular and immune ecto-nucleotidase and an important factor in human pancreatic disease where it is mainly localized to myofibroblast like cells and the vasculature. Although prior descriptive data indicate an altered expression of CD39 and P2-R in pancreatic disease,¹⁶ no analytical studies have so far elucidated whether purinergic signaling participates in ECM turnover and parenchymal repair processes in pancreatitis. The current study shows

the importance of the purinergic system in impacting the extent of fibrosis in an experimental model of chronic pancreatitis.

While there is increased expression of CD39 in the desmoplastic tissues of human pancreatitis and pancreatic cancer,¹⁶ CD39-null mice do not develop substantive pancreatic fibrosis in CIP. CD39 is localized to fibroblast like cells in normal pancreas and CIP in the mouse. During the evolution of experimental pancreatitis, the development of pancreatic fibrosis, atrophy and architecture is significantly less in wt and CD39-null mice. Pancreata of the wt mice show marked parenchymal tissue atrophy and weigh significantly less than those from comparably cerulein treated CD39-null mice. However, endocrine functions of the gland are not impaired in CIP, and blood sugars remain normal in wt and CD39-null pancreatitis.

Fibronectin is more highly expressed on protein level in whole tissue lysates of wt pancreatitis and in wt PSC compared to CD39-null pancreatitis and PSC. Elevated levels of MMP-2 are evident in CD39-null but not wt pancreas lysates, indicating a potential of more active degradation of basement membrane and denatured collagen in the CD39-null system. We did not detect differences in TGF- β 1 regulation. In line with our previous data, this suggests that these protective effects could be related to a Th1 predominant phenotype²² secondary to CD39-null regulatory T immune cell dysfunction. This perturbation might result in the observed high levels of IFN- γ , a cytokine that is known to be angiostatic and displays antifibrotic properties.²⁷

The predominant Th1 phenotype and dysregulation of IFN- γ in CD39-null mice and the resultant attenuated fibrosis might indicate an important role of immune related interactions in pancreatitis impacting upon fibrosis. Increased levels of ATP can inhibit proliferation of peripheral lymphocytes *in vitro*.²⁸ In CD39-null tissues, extracellular and pericellular ATP levels are expected to be increased. This could impair proliferation of CD39-null PSC in experimental chronic pancreatitis, as observed in our studies. Together with the excessive production of circulating IFN- γ , the described differences in P2X7 and IFN- γ expression might, at least in part, explain the difference in extent of fibrosis observed between wt and CD39-null pancreatitis.

Further evidence that CD39 impacts fibrosis was provided by studies of PSC *in vitro*. Primary cultures of CD39-null PSC show a significant deficit in proliferation and are non-responsive *in vitro* to proliferation stimuli from PDGF or profibrotic stimulation from TGF- β . PSC *in vitro* do express CD39 on the mRNA and protein level. CD39-null PSC are viable and appear to differentiate in a comparable manner to wt PSC. CD39L1, which is associated with portal fibroblasts in the liver, is also localized on myofibroblast like cells. PSC also colocalize to CD34 and interestingly, CD39L1 is markedly upregulated in dense fibrotic areas in CP of CD39-null animals, whereas it is only faintly expressed in dense fibrotic areas of pancreatitis in wt animals. Whether this excess of CD39L1 accumulation in the CD39-null environment might be a compensatory effect due to the lack of CD39 remains unclear at the moment.

PSC play a major role in ECM turnover and matrix production. Decreased proliferation of CD39-null PSC and resulting decreased numbers in a CD39-null environment are possibly controlled by primary defects or by altered immunological interactions. The finding of impaired PSC proliferation might be a plausible explanation for the impaired matrix production in CD39-null mice, when compared to the outcomes of wt PSC proliferation and differentiation *in vivo*. Our data demonstrate that CD39-null PSC do not respond adequately to mitogenic stimulation by PDGF and consequently to profibrogenic stimulation by TGF- β , thus resulting in decreased proliferation and impaired procollagen- α 1 production.

CD39 activity is crucial in regulating P2-receptor function by preventing desensitization responses.⁶ This is believed to cause activation of signaling pathways, inducing cell

proliferation. We propose desensitization of P2Y receptors on CD39-null PSC perturbs proliferative responses to growth factor agonists by precluding transactivation processes.²⁹

Further compelling evidence that CD39 and purinergic elements are involved in the complex process of fibrogenesis in comparable cellular systems has been provided by Dranoff et al.⁷ Activation of hepatic stellate cells (HSC) leads to heightened expression of another closely related ecto-nucleotidase, CD39L1, on HSC. Furthermore, addition of extracellular UDP to HSC can triple mRNA levels of procollagen- α 1 that encodes a major constituent of the fibrotic ECM.⁷ CD39L1 expression can be localized in portal fibroblasts adjacent to basolateral membranes of bile duct epithelia⁷

Taken together, the results of the present study indicate that CD39 and purinergic signaling, potentially P2X7, preferentially impact fibrosis and appear to play a major role in modulating ECM remodeling in inflammatory diseases of the pancreas.

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Abbreviations

α -SMA, α -smooth muscle actin
CIP, cerulein induced pancreatitis
CP, chronic pancreatitis
ECM, extracellular matrix
HSC, hepatic stellate cells
IFN- γ , interferon- γ
LC, leukocyte
P2-R, P2 receptor
PDGF, platelet derived growth factor
PSC, pancreatic stellate cells
TGF- β , transforming growth factor- β
wt, wild-type
TIMP-1, tissue inhibitor of metalloproteinases

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Figure 1.

Assessment of pancreatitis. (A) After 6 wks of CIP, significant decreases in pancreas weights of wt versus CD39-null mice were observed (secondary to atrophy of the pancreas, $P<0.005$). (B) Saline injection for 6 wks did not affect pancreas weights (sham treatment). (C, D) Blood amylase activity revealed no differences between cerulein- or sham treated wt and CD39-null mice (normal range of used test, up to 100 IU/L). (E) Heightened levels of IFN- γ were only detected in sera from CD39-null mice after 3 wks of pancreatitis induction ($P<0.05$). (F) Blood sugar levels remained normal and did not differ between the treatment groups.

Figure 2.

Western analyses. Protein expression was determined from baseline pancreas without treatment, after 3 wks and 6 wks of CIP. (A) Representative samples showing high induction of CD39 at 3 and 6 wks of CIP in wt mice. CD39-null mice show increased protein expression of CD39L1 and P2X7 (at 3 wks). P2Y2 was upregulated after 6 wks. MMP-2 was significantly upregulated but only in CD39-null mice. TGF- β 1 was equally upregulated at 3 wks of CIP. CD45 was predominantly expressed in CD39-null mice at 3 wks of CIP treatment. (A) Fibronectin was highly upregulated at 6 wks treatment, more prominently expressed in wt mice. Each lane (40 μ g total protein/lane) represents one pancreas sample. Shown is one representative individual mouse per group (baseline pancreas, CIP at 3 and 6 wks time,

respectively). (B) Protein lysates from primary cultured PSC showed high expression for CD39. P2X7 expression was more prominent in wt cells when compared to CD39-null PSC. Protein levels of fibronectin were elevated in wt PSC versus CD39-null PSC.

Figure 3.

Pancreas morphology. (A) Pancreas architecture in sham treated remained unchanged in wt and CD39-null mice. (B) Panels show representative Masson's trichrome staining sections from wt and CD39-null mice. Histological parameters such as fibrosis (C) ($P<0.02$) and atrophy (D) ($P<0.02$) revealed significant differences between wt and CD39-null pancreases.

Figure 4.

Immunohistochemistry in cerulein induced pancreatitis. (A, B) Panels show representative immunohistochemical analyses of CD39 and P2X7 in tissues of wt and CD39-null mice after 6 wks of CIP. Normal pancreas of sham treated wt and CD39-null mice did not reveal any signs of significant architectural changes after saline treatment. (B, D) There was no reactivity for CD39 in CD39-null mice. During pancreatitis, the density of CD39 positive staining increased and localized to endothelial and cells in the interacinar and interseptal space (insert Figure 3C), that share homologies with myofibroblast like cells and resembling PSC in localization and morphology. (E, F) P2X7 is mainly localized to immune cells (LC = leukocyte) and elements of the vasculature.

Figure 5.

Immunohistochemistry and immunofluorescence in chronic pancreatitis. (A, B) CD34, a myofibroblast- and stromal cell marker localized within the periacinar and periductular areas. CD39 is colocalized with CD34. (C, D) The double positive cells display a distinct morphology, are single cells with spindle like protrusions and comparable with the described phenotype and morphology of PSC. (E, F) CD39L1 was colocalized to CD34 in the rarely encountered areas of dense fibrosis in CD39-null mice (insert Figure 4F), whereas in dense fibrotic areas of wt mice, co-localization was only minimal.

Figure 6.

Phenotype and differentiation of PSC in primary cultures. (A, B) CD39-null PSC displayed a highly significant proliferation deficit in primary culture compared to wt PSC ($P < 0.02$). (A) Stimulation of CD39-null PSC with PDGF (5 ng/ml) did not lead to increased proliferation in CD39-null PSC, whereas wt PSC responded to PDGF. (C) Elevated concentration of ATP levels (10 and 50 μ mol ATP but not 150 and 400 μ mol) in culture media significantly increased proliferation of wt PSC ($P < 0.05$). (D) Significant differences in mRNA expression profile for CD39 and P2X7 in cultures of wt PSC compared to CD39-null PSC. CD39-null PSC were viable and differentiated in a comparable manner to wt PSC, as indicated by equal expression of α -SMA on mRNA (E) and morphologically (F). Morphology of PSC revealed high positive

staining for α -SMA and desmin in cultured PSC. (F) Differentiation and viability were comparable in wt and CD39-null PSC.

Figure 7.

Proliferation defect in CD39-null PSC. In general, a lack of plasticity was noted in CD39-null PSC responses to growth factors and TGF- β . Wt and CD39-null PSC were stimulated with PDGF (5ng/ml) or TGF- β 1 (2ng/ml), (A) The mRNA expression profiles reached moderate levels of CD39 expression in wt PSC. (B) CD39L1 levels of expression in wt (and to a lesser extent in CD39-null PSC) decreased when stimulated with PDGF or TGF- β 1. P2X7 expression in wt PSC was significantly increased when compared to CD39-null PSC ($P < 0.03$). (C) P2X7 levels decreased during stimulation with PDGF and TGF- β 1. (D) Levels of P2Y2 were comparable during stimulation experiments. (E) At baseline conditions and under stimulation with PDGF, α -SMA remained expressed at comparable levels. Procollagen- α 1 production

following PDGF stimulation was significantly decreased in CD39-null PSC. (F) Most strikingly, procollagen- α 1 expression in wt PSC significantly increased when stimulated with the profibrogenic cytokine TGF- β 1 ($P<0.03$). This defect suggests non-responsiveness of and probable defect in collagen production in the CD39-null PSC.

Table 1
Quantification of mRNA transcripts in pancreatic lysates of wild-type and CD39-null mice

Tissue	mRNA	wild-type		CD39-null		P value
		Mean	SD	Mean	SD	
A. Baseline Pancreas	CD39*	148	44	3.5	2.9	P < 0.05
	CD39L1	118	26	67.4	49.4	
	P2X7	11.6	7.9	14.1	9.8	
	P2Y2	0.8	0.9	3.4	2.3	
	CD31	30	8.9	46.6	30.5	
	CD34	166	66.5	167	152	
	CD45	332	249	464	302	
	IFN-γ	2.23	0.32	0.54	0.32	
	Procollagen-α	4,964	3,319	1,447	827	
	α-SMA	2.2	1.1	11.1	10.3	
	TGF-β1	56.7	42.0	24.5	20.7	
	MMP-2	7.8	8.8	6.8	13.3	
	MMP-9	4.5	4.9	3.1	2.7	
	MMP-13	1.7	2.0	0.30	0.15	
	TIMP-1	32.4	16.9	43.7	20.6	
B. CIP (3 wks)	CD39**	1,420	487	32.2	6.7	P < 0.01
	CD39L1*	655	175	986	258	P < 0.05
	P2Y2**	11.9	3.9	24.2	4.1	P < 0.01
	IFN-γ*	38.5	21.8	138	114	P < 0.05
	TIMP-1*	1,546	646	3,795	1,627	P < 0.05
C. CIP (6 wks)	CD39**	1,640	454	27.9	14.7	P < 0.01
	MMP-2*	5,600	7,206	2,399	5,343	P < 0.05
	MMP-9*	312	342	114	83.3	P < 0.05

Mean values shown are normalized to 18S mRNA levels.
CIP, cerulein-induced pancreatitis; SD, standard deviation; P values indicate differences between wild-type and CD39-null animals. (bold indicative of significance < 0.05)