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BASIC AND TRANSLATIONAL—PANCREAS

Release of Cathepsin B in Cytosol Causes Cell Death in Acute Pancreatitis

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BACKGROUND & AIMS: Experimental studies in acute pancreatitis (AP) suggest a strong association of acinar cell injury with cathepsin B–dependent intracellular activation of trypsin. However, the molecular events subsequent to trypsin activation and their role, if any, in cell death is not clear. In this study, we have explored intra-acinar events downstream of trypsin activation that lead to acinar cell death. **METHODS:** Acinar cells prepared from the pancreas of rats or mice (wild-type, trypsinogen 7, or cathepsin B–deleted) were stimulated with supramaximal cerulein, and the cytosolic activity of cathepsin B and trypsin was evaluated. Permeabilized acini were used to understand the differential role of cytosolic trypsin vs cytosolic cathepsin B in activation of apoptosis. Cell death was evaluated by measuring specific markers for apoptosis and necrosis. **RESULTS:** Both in vitro and in vivo studies have suggested that during AP cathepsin B leaks into the cytosol from co-localized organelles, through a mechanism dependent on active trypsin. Cytosolic cathepsin B but not trypsin activates the intrinsic pathway of apoptosis through cleavage of bid and activation of bax. Finally, excessive release of cathepsin B into the cytosol can lead to cell death through necrosis. **CONCLUSIONS:** This report defines the role of trypsin in AP and shows that cytosolic cathepsin B but not trypsin activates cell death pathways. This report also suggests that trypsin is a requisite for AP only because it causes release of cathepsin B into the cytosol.

Keywords: Cytosolic Cathepsin B; Apoptosis; Necrosis; Bid Cleavage.

Acute pancreatitis (AP) is an inflammatory disease of the pancreas that originates within the pancreatic acinar cells.[1,2] We and others have shown that pancreatitis begins with co-localization of lysosomes and zymogens.[3,4] After co-localization of these 2 subcellular compartments, the lysosomal hydrolytic enzyme cathepsin B activates trypsinogen to form active trypsin.[2,5,6] The activated trypsin within the acinar cell has long been held to be the major player responsible for the acinar cell damage in AP. Although a large body of evidence from studies conducted by us and others suggests that injury to the pancreas culminates in either apoptosis or necrosis of acinar cells,[7–9] the events downstream of co-localization and the primary trigger that initiates the cell death pathways still remain speculative. On the other hand, it is well proven that cathepsin B is capable of causing apoptosis in hepatocytes and a number of other cell types.[10,11]

In this article, we investigate the cell death trigger in experimental pancreatitis and evaluate the role of cathepsin B beyond activation of trypsinogen. We show that active trypsin is required for leakage of cathepsin B into the acinar cell cytosol from the co-localized organelles. Once released into the cytosol, cathepsin B, but not trypsin, activates the apoptotic cascade. Our data also suggest that although a small amount of cathepsin B released into the cytosol can activate apoptosis, massive release of cathepsin B promotes necrosis.

Materials and Methods

Materials

Male Wistar rats and wild-type mice (C57BL/6) were purchased from Charles River Laboratories (Wilmington, MA), cathepsin B knockout ($CTSB^{-/-}$) mice in C57BL6 background were kindly provided by Dr G. J. Gores (Mayo Clinic, Rochester, MN), and we generated the trypsinogen isoform-7 gene knockout ($T7^{-/-}$) mice in C57BL/6.[12]

In Vitro Experimental Protocol

Pancreatic acini were prepared from male Wistar rats, WT C57BL6 mice, $CTSB^{-/-}$ mice, or $T7^{-/-}$ mice by collagenase digestion, as previously described.[13] The acini were suspended in oxygen-saturated HEPES Ringer buffer (pH 7.4) with 0.1% bovine serum albumin and stimulated with supramaximal or maximal doses of caerulein, cholecystokinin (CCK)-JMV180, or carbachol, or incubated with sphingosine, depending on the experiment. In experiments evaluating the

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Abbreviations used in this paper: **AP, acute pancreatitis; CBKO, cathepsin B knockout; CCK, cholecystokinin; CP, chronic pancreatitis; HSP, heat shock protein; LDH, lactate dehydrogenase; LLOMe, Leu-Leu-OMe; PI, phosphatidylinositol; RIP, receptor-interacting protein kinase; SLO, streptolysin-O; T7KO, trypsinogen-7 knockout; WT, wild-type.**

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effect of cathepsin B inhibition, phosphatidylinositol (PI)3 kinase inhibition, trypsin inhibition, calcium chelation, and Bid cleavage, acini were pretreated by the cell-permeable cathepsin B inhibitor CA074-me (10 μmol/L), cell-nonpermeable cathepsin B inhibitor CA074 (20 μmol/L), trypsin inhibitor benzamidine (1 mmol/L), PI3 kinase inhibitors Wortmannin (20 nmol/L) or Ly294002 (50 μmol/L), or calcium chelator BAPTA-AM (20 μmol/L) for 20 minutes before stimulation with caerulein. Heat shock protein (HSP)-70 overexpression in animals was achieved by subjection to thermal stress (42°C for 20 minutes) or sodium arsenite (5 mg/kg intraperitoneally) treatment and acini were prepared 12 hours after the treatment. Other methods are described in the Supplementary Materials and Methods section.

Results

Cathepsin B Is Released Into the Cytosol During Pancreatitis

To evaluate if cathepsin B is released into the cytosol during AP, rat pancreatic acini, after appropriate treatment, were fractionated into cytosolic and membrane fractions using the streptolysin-O (SLO) permeabilization method and cytosolic cathepsin B was measured by Western blot and enzymatic assay. We observed a marked increase in cytosolic cathepsin B after supramaximal stimulation with caerulein, both by Western blot (Figure 1*A*) and enzymatic assay (Figure 1*B*). Similarly, a high level of cathepsin B activity was seen in the cytosol of acini stimulated with supramaximal carbachol (Figure 1*B*). Treatment of acini with maximal doses of caerulein or carbachol, or maximal or supramaximal doses of CCK-JMV180 did not cause an increase in cytosolic cathepsin B activity (Figure 1*B*). Supramaximal caerulein also caused an increase in the cytosolic activity of another lysosomal enzyme aryl sulfatase (Figure 1*C*). To ensure that the increase in cytosolic cathepsin B activity was not the result of differential sensitivity of the plasma membranes of control, maximal, and supramaximal caerulein-treated acini to SLO-induced permeabilization, we normalized cytosolic cathepsin B activity to lactate dehydrogenase (LDH) content. As seen in Supplementary Figure 1, supramaximal cerulein stimulation led to increased cytosolic cathepsin B activity even when normalized to LDH. We also ensured that this increase was not an artifact caused by contamination of the cytosol with endosomes, because endosomes could be another possible source of cathepsin B, by evaluating the presence of Rab7 in cytosol (Supplementary Figure 2).

To evaluate this phenomenon in vivo, we prepared pancreatic acini from rats pretreated with supramaximal caerulein or L-arginine, fractionated the acinar cells into cytosol and membrane fractions using the SLO method, and evaluated for cathepsin B in the cytosol. In both in vivo models, there was a significant increase in cytosolic cathepsin B activity (Figure 1*D*). These findings were confirmed on immunofluorescence where we observed punctate staining for cathepsin B in the acinar cells of saline-treated rats (Figure 1*E*), suggesting its localization inside lysosomes, whereas in rats with caerulein pancreatitis we observe markedly more diffuse staining for cathepsin B, suggesting release of cathepsin B into the cytosol. Taken together, these observations suggest that during AP, both in vitro and in vivo, lysosomal enzyme-containing organelles in the acinar cells become fragile and release cathepsin B into the cytosol.

To evaluate the relevance of cytosolic cathepsin B during pancreatitis in human disease, we performed cathepsin B immunostaining on normal pancreas obtained from healthy deceased donors and compared it with that in patients with chronic pancreatitis (CP). As seen in Figure 1*F*, in normal pancreas cathepsin B stains in punctate fashion, suggesting intralysosomal localization. In patients with CP, in many acinar cells cathepsin B is present in a diffuse fashion, suggesting its presence in cytosol.

Cytosolic Cathepsin B Released Into the Cytosol of the Caerulein-Stimulated Pancreatic Acinar Cells Arises From Co-localized Organelles and Not Lysosomes

In contrast to lysosomes, by definition co-localized organelles have both lysosomal enzymes and digestive enzymes such as amylase. Furthermore, co-localized organelles are the only intracellular site where active trypsin is present. As shown in Figure 2*A* and B, in the supramaximal caerulein-stimulated acini, there was a significant increase in the cytosolic amylase and trypsin activity. Furthermore, inhibition of co-localization by pretreatment with either Wortmannin or Ly294002[14] significantly reduced the cytosolic amylase, trypsin, and also cathepsin B activity (Figure 2*C*). HSP70 overexpression by thermal stress and sodium arsenite treatment also has been shown to inhibit lysosome-zymogen co-localization.[15] As shown in Figure 2*D*, cytosolic cathepsin B in caerulein-stimulated pancreatic acini from rats pretreated with thermal stress or sodium arsenite also was significantly less than that from untreated rats. Collectively, these observations suggest that cathepsin B is released from co-localized organelles into the cytosol after supramaximal stimulation with caerulein.

We also have previously shown that calcium is required for co-localization. Thus, we evaluated the impact of attenuation of cytosolic calcium on the release of cathepsin B into the cytosol. As shown in Figure 2*E*, pretreatment of caerulein-stimulated acini with the calcium chelator BAPTA-AM led to a significant decrease in cytosolic cathepsin B activity.

Trypsin Is Required for Release of Cathepsin B From Co-localized Organelles Into the Cytosol

We next evaluated the role of trypsin in the release of cathepsin B into the cytosol. For this we compared the impact of supramaximal caerulein stimulation on release of cathepsin B into the cytosol of pancreatic acini in wild-type (WT) and trypsinogen-7 knockout (T7KO) mice. As seen in