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Release of cathepsin-B in cytosol causes cell death in acute pancreatitis

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Abstract

BACKGROUND & AIMS—Experimental studies in acute pancreatitis (AP) suggest 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 have not been studied. In this study, we have explored, for the first time, intra-acinar events downstream of trypsin activation which 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 caerulein and 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 suggest that during AP cathepsin-B leaks into the cytosol from co-localized organelles, through a mechanism dependent on active trypsin. Cytosolic

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b) Acquisition of data. RT, HZ, VD, UB, AS, RS, SKG, SB, PG, ZY, AD, JG, HC.

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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 is the first report which defines the role of trypsin in AP and demonstrates that cytosolic cathepsin-B but not trypsin activates cell death pathways. This is also the first report to suggest that trypsin is requisite for AP only because it causes release of cathepsin-B into the cytosol.

Keywords

Cytosolic cathepsin-B; apoptosis; necrosis; Bid cleavage

Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas which 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 two 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. While 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 cells⁷⁻⁹, 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 paper, we investigate the cell death trigger in experimental pancreatitis and evaluate the role of cathepsin-B beyond activation of trypsinogen. We show for the first time 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, while 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. GJ. Gores, Mayo Clinic, Rochester, MN, while trypsinogen isoform-7 gene knockout (T7^{-/-}) mice in C57BL/6 were generated by us¹².

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 described¹³. 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, CCK-JMV180, or carbachol, or incubated with sphingosine depending on the experiment. In experiments evaluating the effect of cathepsin-B inhibition, PI-3K inhibition, trypsin inhibition, calcium chelation, and Bid cleavage, acini were pre-treated by the cell permeable cathepsin-B inhibitor CA074-me(10 μ M), or cell non-permeable cathepsin-B inhibitor CA074(20 μ M), or trypsin inhibitor benzamidine(1mM), or PI3-K inhibitors Wortmannin(20nM) or Ly294002(50 μ M), or calcium chelator BAPTA-AM(20 μ M) for 20 min prior to stimulation with caerulein. HSP-70 overexpression in animals was achieved by subjecting to thermal stress (42° C for 20mins) or sodium arsenite (5mg/kg i.p.) treatment and acini were prepared 12 hours after the treatment. Other methods are described in the supplementary data section.

Results

Cathepsin-B is released into the cytosol during pancreatitis

To evaluate if cathepsin-B is released into the cytosol during acute pancreatitis (AP), rat pancreatic acini, after appropriate treatment were fractionated into cytosolic and membrane fractions using Streptolysin-O (SLO) permeabilization method and cytosolic cathepsin-B was measured by Western blotting and enzymatic assay. We observed a marked increase in cytosolic cathepsin-B after supramaximal stimulation with caerulein, both by Western blotting (Figure-1A) and enzymatic assay (Figure-1B). Similarly, high level of cathepsin-B activity was seen in the cytosol of acini stimulated with supramaximal carbachol (Figure-1B). Treatment of acini with maximal doses of caerulein or carbachol or maximal or supramaximal doses of CCK-JMV180 did not cause increase in cytosolic cathepsin-B activity (Figure-1B). Supramaximal caerulein also caused increase in the cytosolic activity of another lysosomal enzyme aryl sulfatase (Figure-1C). To ensure that the increase in cytosolic cathepsin-B activity was not due to 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 LDH content. As seen in supplementary figure-1, supra-maximal caerulein stimulation leads to increased cytosolic cathepsin-B activity even when normalized to LDH. We also ensured that this increase was not an artefact due to contamination of the cytosol with endosomes, as endosomes could be other 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 pre-treated with supramaximal caerulein or L-arginine, fractionated the acinar cells into cytosol and membrane fraction using the SLO method and evaluated for cathepsin-B in the cytosol. In both *in-vivo* models, there was significant increase in cytosolic cathepsin-B activity (Figure-1D). These findings were confirmed on immunofluorescence where we observed punctate staining for cathepsin-B in the acinar cells of saline treated rats (Figure-1E), 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 became 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-1F, in normal pancreas cathepsin-B stains in punctate fashion suggesting intra-lysosomal localization. In patient with CP, in many acinar cells cathepsin-B is present in diffuse fashion suggesting 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-2A and -2B, in the supramaximal caerulein-stimulated acini, there was a significant increase in the cytosolic amylase and trypsin activity. Furthermore, inhibition of co-localization by pre-treatment with either Wortmannin or Ly294002¹⁴ significantly reduced the cytosolic amylase, trypsin and also cathepsin-B activity (Figure-2C). HSP70 over-expression by thermal stress and sodium arsenite treatment has also been shown to inhibit lysosome-zymogen co-localization¹⁵. As shown in Figure-2D, cytosolic cathepsin-B in caerulein-stimulated pancreatic acini from rats pretreated with thermal stress or sodium arsenite was also significantly less than that from untreated rats. Collectively, these observations suggest that cathepsin-B is released from co-localized organelles into the cytosol following supramaximal stimulation with caerulein.

We have also previously demonstrated 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-2E, pre-treatment 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 supra-maximal caerulein stimulation on release of cathepsin-B into the cytosol of pancreatic acini in WT and Trypsinogen-7 knock-out (T7KO) mice. As seen in figure-2F, treatment of acini from WT mice with supra-maximal caerulein leads to increased cytosolic cathepsin-B. However, supra-maximal caerulein induced release of cathepsin-B into the cytosol was not observed in T7KO mice (Figure-2F) suggesting that trypsin plays a role in release of cathepsin-B from the co-localized organelles. Similarly, pre-treatment of caerulein-stimulated rat acini with the trypsin inhibitor benzamidine led to marked reduction of cytosolic cathepsin-B activity (Supplementary figure-3), again supporting the conclusion that trypsin is required for cathepsin-B release from co-localized organelles.

Cytosolic Cathepsin-B leads to acinar cell apoptosis during pancreatitis

We next evaluated the role of cytosolic cathepsin-B and trypsin in acinar cell apoptosis. Supramaximal caerulein stimulation of rat pancreatic acini led to marked increase in apoptosis as suggested by increased caspase-3 activity (Figure-3A), increased green

fluorescence on ApoTRACE staining (Figure-3B) and by a significant increase in ApoTRACE dye extraction (Figure-3C). Increased caspase-3 activation was observed also in acini prepared from animals treated with either supramaximal caerulein or L-arginine (Figure-3D). Inhibition of cathepsin-B with CA074-me or trypsin with benzamidine pretreatment reduced ApoTRACE dye uptake (Figures-3E) and caspase-3 activation (Figure-3F), suggesting that both cathepsin-B and trypsin are required for acinar cell apoptosis. Next we further confirmed the role of cytosolic cathepsin-B in activation of apoptosis. As a proof of principle, to prove that cytosolic cathepsin-B is involved in acinar cell apoptosis, we used lysosomotropic detergent sphingosine which is known to cause release of cathepsin-B and other lysosomal-enzymes to the cytosol¹⁶. As shown in supplementary data (Supplementary Figure-4A and -4B), with increasing doses of sphingosine, there was an increase in caspase-3 activation that was significantly inhibited by CA074-me pre-treatment.

To further evaluate whether cytosolic cathepsin-B or trypsin can activate apoptosis, we measured caspase-3 activation after treating SLO-permeabilized rat acini, which were not stimulated with caerulein, with different doses of exogenous cathepsin-B and trypsin (simulating presence of cathepsin-B or trypsin in cytosol). The activity of exogenous Cathepsin-B and trypsin added were comparable to that detected in cytosol after supramaximal caerulein stimulation of rat acini. As shown in Figure-4, there was no caspase-3 activation on addition of trypsin to the cytosol at any dose in contrast to dose dependent caspase-3 activation seen on addition of cathepsin-B to the cytosol suggesting that cytosolic cathepsin-B, but not trypsin, causes apoptosis.

We further evaluated the role of cathepsin-B and trypsin in apoptotic acinar cell death using WT, cathepsin-B^{-/-} (CBKO) and T7^{-/-} (T7KO) mice. As shown in Figure-5A, cell viability in WT acini decreased by ~60% upon 3h of stimulation with 10⁻⁷M caerulein while 10⁻¹⁰ M caerulein did not cause cell death. Pretreatment of acinar cells with CA074-me, a cathepsin-B inhibitor, completely blocked caerulein-induced cell death. Similarly, there was no significant supramaximal caerulein-induced cell death in CBKO or T7KO mice acini. Caspase-3 activation accompanied the reduced cell viability of WT mice acini exposed to 10⁻⁷ M caerulein for 3h (Figure-5B) while no elevated caspase-3 activity was detected with maximal caerulein stimulation (Figure-5B) or by supramaximal caerulein exposure in either CA074-me-pretreated WT mice or in CBKO or T7KO mice acini.

Given the earlier findings that cathepsin-B release into the cytosol induces apoptosis during pancreatitis, we further hypothesized that re-introduction of cathepsin-B into the cytosol of permeabilized CBKO pancreatic acini, by adding exogenous cathepsin-B, may convert its apoptosis-protective to pro-apoptotic phenotype. To test this, WT and CBKO acini were permeabilized with SLO. In some cases, the cells were pre-stimulated with 10⁻⁷ M caerulein prior to SLO treatment. The cells were then exposed to bovine cathepsin-B for 3h. Apoptosis was evaluated by measuring caspase-3 activation. As shown in Figure-5C, addition of exogenous bovine cathepsin-B to SLO permeabilized CBKO acini activated caspase-3 to a comparable level as it did in SLO-permeabilized WT acini. Supramaximal caerulein stimulation combined with saturated concentration of bovine cathepsin-B did not further enhance caspase-3 activity. Conversely, in the absence of exogenous cathepsin-B, caspase-3

remained inactive in SLO-permeabilized CTSB^{-/-} acini, indicating active cathepsin-B in the cytosol induces apoptosis.

We further reasoned that loss of function of cathepsin-B by introducing cathepsin-B inhibitor into the cytosol of WT acini would inhibit apoptosis (Figure-5D). To investigate this, WT acini were permeabilized with SLO, or stimulated by caerulein before incubation with SLO, then incubated with CA074, a cell non-permeable cathepsin-B inhibitor, prior to bovine cathepsin-B exposure. As shown in Figure-5D, caspase-3 was significantly activated by addition of exogenous bovine cathepsin-B to permeabilized WT acini, whether treated or untreated with caerulein. CA074 treatment completely abrogated caspase-3 activation in similarly treated cells. This demonstrates that cytosolic cathepsin-B inhibition protects pancreatic acini against cathepsin-B-induced apoptosis.

Cathepsin-B-induced apoptosis of acinar cells is mitochondrial pathway-dependent

To gain insight into the mechanisms by which cytosolic cathepsin-B induces apoptosis, we first examined whether caerulein induces pro-apoptotic Bid cleavage and Bax activation. *In vivo* decreased precursor Bid and increased tBid levels were observed after supramaximal caerulein stimulation (50µg/kg X 3 injection followed by sacrifice at 4h) in WT mice but not in CB-KO mice (Figure-6A). Quantification of Bid cleavage from at least three independent experiments is shown in Figure-6B. Similarly, *in vitro* supramaximal caerulein stimulation led to bid cleavage with increased levels of truncated Bid (tBid) and reduced levels of precursor Bid in WT mouse pancreatic acinar cells, no Bid cleavage was observed in CB-KO mice (supplementary figure-5.) Bax activation was detected using an anti-active Bax (6A7) conformation-dependent antibody. While supramaximal caerulein stimulation caused Bax activation (Figure-6C) in WT mouse pancreatic acinar cells, no Bax activation was observed in cells prepared from CBKO mice (Figure-6C).

Translocation of cytochrome-C from mitochondria to the cytosol

Western blotting for cytochrome-c in the membrane and cytosolic fractions of caerulein-stimulated acini showed an increase in cytosolic cytochrome-c with a corresponding decrease in the membrane fraction (Figure-6D) suggesting release of cytochrome-c from the mitochondria into the cytosol. The release of cytochrome-c was prevented on pretreatment with CA074-me. To further evaluate the role of cathepsin-B induced cytochrome C release in apoptosis we added extrinsic cathepsin-B to the permeabilized acini with neutralizing anti-cytochrome c or control isotype antibody. As seen in figure-6D, neutralization of the cytochrome c prevents cathepsin-B induced caspase 3 activation.

Role of cytosolic cathepsin-B in acinar cell death through necrosis

Literature¹⁷⁻¹⁹ suggest that while small amount of cytosolic cathepsin-B can cause cell death by apoptosis, large amount of cytosolic cathepsin-B can shift the cell death pathway towards necrosis. To evaluate whether such a phenomenon exist in acinar cells, we treated acinar cells from C57BL/6J mice with increasing dose of Leu-Leu-OMe (LLOMe). LLOMe accumulates in lysosomes and after hydrolysis the product formed causes the lysosomes to rupture resulting in release of cathepsin-B in cytosol. As seen in figure-6E, LLOMe leads to release of cathepsin-B into the cytosol of acinar cells in a dose dependent fashion.

Intriguingly, at lower doses, which cause a small increase of cathepsin-B into the cytosol, we observe that there is activation of caspase-3 suggesting apoptotic cell death. However, at higher doses, when there is large amount of cathepsin-B in the cytosol, there is in fact a decrease in caspase-3 activation and increase in LDH release suggesting an increase in cell death by necrosis. Studies in non-pancreatic system suggest that during necrosis receptor interacting protein kinase-1 (RIP1) and -3 come together to form a complex. We thus evaluated whether LLOMe treatment of acinar cells leads to RIP-1/RIP-3 interaction at higher doses. RIP3 was immunoprecipitated from LLOMe treated acinar cell lysates and immunoprecipitates were immunoblotted for RIP1 and RIP3. As seen in supplementary figure=6, we do not observe any RIP1 and RIP3 interaction at low dose of LLOMe, where apoptosis is the dominant mechanism of cell death. However, at higher doses of LLOMe, we observed a dose dependent RIP1/RIP3 interaction suggesting potential role of RIP1/RIP3 in necrotic cell death in pancreatic acinar cells.

We repeated these experiments in mouse pancreatic acinar cell line 266-6. 266-6 cell line is derived from the mouse tumor induced with elastase I/SV-40 T-antigen fusion gene. 266-6 cells retain a partially differentiated phenotype and express several digestive-enzymes²⁰. They respond to carbachol and CCK and have been used to investigate the physiology and pathology of the pancreas²¹. We observed that high dose of LLOMe treatment leads to significant cell death in 266-6 cell line through necrosis as indicated by increased LDH release (Supplementary figure-7). Furthermore, pre-treatment with cathepsin-B inhibitor CA074-me markedly inhibits cell death through necrosis confirming the involvement of cathepsin-B in cell death through necrosis (Supplementary figure-7). Similarly, while higher dose of LLOMe is able to induce necrosis in acini from wild type mice, we did not observe any necrosis in cathepsin-B knockout mice when treated with LLOMe indicating massive release of cathepsin-B in the cytosol can cause cell death through necrosis (Figure-6F).

Discussion

Co-localization of lysosomes and digestive zymogens within the pancreatic acinar cells is a major early event in acute pancreatitis (AP). We have previously shown that co-localization occurs within 30min of noxious stimulation to the pancreas and that this is followed by activation of trypsinogen to trypsin by the lysosomal cysteine protease cathepsin-B within the co-localized organelles³. Subsequently pancreatic injury is observed in the form of acinar cell apoptosis or necrosis. However, the events downstream of co-localization that culminate in acinar cell injury have been unclear. In the present paper, we investigated the events downstream of co-localization that trigger acinar cell injury in AP and have shown, for the first time, that cytosolic cathepsin-B causes acinar cell death during AP. Our results demonstrate that during AP, contents of co-localized organelles, including lysosomal-enzymes and digestive enzyme zymogens, are released into the cytosol of the acinar cells. Once in the cytosol, cathepsin-B but not active trypsin, activates the cell death pathways. Intriguingly, trypsin is required for release of the contents of co-localized organelles including cathepsin-B into the cytosol, thus explaining the finding that inhibition of either cathepsin-B or trypsin prevents acinar cell death.

Cytosolic cathepsin-B has been shown to induce apoptosis in a number of cell lines^{10, 11}. Thus we hypothesized that cathepsin-B in the cytosol could be a possible trigger for apoptosis in acinar cells. We initially examined the presence of cathepsin-B in the cytosol both *in-vitro* and *in-vivo* after induction of supramaximal caerulein-induced pancreatic injury. We found that active cathepsin-B was present in the cytosol after stimulation with agents which cause pancreatic injury, e.g. supramaximal caerulein and carbachol, and not by agents which do not cause acinar cell injury, e.g. maximal dose of caerulein and carbachol and maximal or supramaximal dose of CCK-JMV-180. Carbachol, a muscarinic agonist that is known to produce acinar cell injury only at supramaximal doses²². CCK-JMV-180 is a partial CCK agonist that stimulates pancreatic amylase secretion to a degree equivalent to that produced by a maximal concentration of CCK, but without causing pancreatic injury even at a supramaximal concentration²³. In an *in-vivo* setting, we demonstrated using two different models (to rule out model specific effect) that during AP there is release of cathepsin-B into the cytosol. To extend these findings to humans, we compared the cathepsin-B immunostaining in pancreatic sections from normal deceased donors and patients with CP and observed that in patients with CP many acinar cells demonstrate presence of cathepsin-B in the cytosol. We believe that in patients with CP there is ongoing acinar cell injury and the acinar cells with cytosolic cathepsin-B are undergoing cell death.

Having shown that there is leakage of cathepsin-B into the cytosol following noxious stimuli, we next elucidated the source of this cytosolic cathepsin-B. This issue was approached by two different approaches. Co-localized organelles have both lysosomal and zymogen contents. Therefore, we first evaluated the cytosolic activities of the zymogen contents (trypsin and amylase), with the premise that if the cytosolic cathepsin-B is originating in the co-localized organelles, there should also be release of trypsin and amylase into the cytosol. Second, we evaluated the effect of inhibition of co-localization by the PI3 kinase inhibitors Wortmannin and Ly294002 and by induction of HSP70 expression^{14, 15, 24} on the release of cathepsin-B into the cytosol. Increased cytosolic activities of trypsin and amylase followed by a significant reduction after inhibition of co-localization by PI3 kinase inhibition and HSP70 over-expression proved that the cytosolic cathepsin-B was released from fragile co-localized organelles. We used different PI3 kinase inhibitors (Wortmannin and Ly294002) because they act by different mechanisms thus ruling out non-specific effect of one particular drug. Prolonged elevated cytosolic Ca²⁺ during pancreatitis is also important for co-localization of zymogens and lysosomal contents and our data suggest that attenuation of calcium by BAPTA, prevents Cathepsin-B release into the cytosol again suggesting that cytosolic Cathepsin-B is arising from co-localized organelles.

While it is clear that during AP the zymogen-enzymes and lysosomal-enzymes come together, the nature of and mechanism of formation of co-localized organelles is a matter of debate. There are at least three possible mechanisms of this process 1) fusion of zymogen granules with lysosomes; 2) perturbation of intracellular trafficking of zymogen granules and lysosomal hydrolases; and 3) endocytic vacuole formation through uptake of secreted digestive-enzymes by acinar cells via endocytosis and fusion of these endosomes with lysosomes. Studies have also suggested that cytoplasmic vacuoles induced in pancreatic acinar cells by experimental pancreatitis are possibly of autophagic origin^{25, 26}. It appears that well-functioning autophagy machinery is important in homeostasis of pancreas and its

impairment can lead to pancreatic injury in the form of atrophy, fibrosis and chronic pancreatitis^{27, 28}. Mareninova et al²⁶ have suggested that during AP there is increased formation of autophagosomes but at the same time there is decreased autophagic efficiency. The authors also demonstrated that during pancreatitis lysosomal-enzymes and zymogens come together, confirming our findings of co-localization. However, authors also demonstrated that there is co-localization of the lysosomal-enzymes with autophagosome marker LC3. The authors also demonstrate that inhibition of autophagy actually inhibits trypsinogen activation. This suggests that the co-localized organelles described by us could be autophagic vacuoles observed by others. Our studies here raise the possibility that cytosolic Cathepsin-B in fact arises from autophagic vacuoles formed in pancreatitis and this possibility will be explored in future studies.

We and others have earlier shown that trypsinogen is activated to active trypsin by cathepsin-B within the co-localized organelles⁶. However, the role of this activated trypsin in acinar cell death and AP as such is a matter of intense discussion. Using T7KO mice we have shown that trypsinogen activation is important, though partially, for acinar cell injury. We have also shown that inflammation during AP is independent of trypsin formation. Others have also addressed the role of trypsin during AP. Gaiser et al²⁹ used a novel transgenic model where endogenously activated trypsinogen was conditionally expressed in the pancreatic acinar cells. This activated trypsin was targeted to the zymogen compartment. The authors observed that these mice demonstrate changes of pancreatitis including acinar cell injury, caspase-3 activation and NF κ B activation suggesting the role of trypsin in AP. Our data also suggest that trypsin is important for cell death during AP. While Gaiser et al do not address the events subsequent to trypsin activation which lead to activation of cell death pathways, the current study primarily deals with events subsequent to trypsin activation. The current study demonstrates that in the absence of trypsin, either in T7KO mice or by pharmacologic inhibition of trypsin, the release of cathepsin-B into the cytosol is prevented. These data suggest that active trypsin within the co-localized organelles plays a role in making the membranes fragile, through which the cathepsin-B and other enzymes leak out into the cytosol. Furthermore, our data demonstrates that only a fraction of cathepsin-B, amylase, active trypsin and arylsulfatase are released from the co-localized organelles into the cytosol. Our previous studies have suggested that only a portion of lysosomes and zymogen granules come together and co-localize. The current data suggests that only a fraction of the active trypsin from the co-localized organelles is released into the cytosol suggests that only some of the co-localized organelles get permeabilized during AP. The factors which determine which co-localized organelles become leaky and release their content into the cytosol are not known and are the area of future investigations.

Our next question was to evaluate the role of cytosolic cathepsin-B in induction of apoptosis of acinar cells. We demonstrated apoptosis following supramaximal caerulein stimulation and inhibition of apoptosis by pre-treatment with cathepsin-B and trypsin inhibitors. At this point, it appeared that both cathepsin-B and trypsin could be responsible for acinar cell apoptosis. Another approach that we undertook to assess the role of cathepsin-B in the induction of apoptosis was to simulate presence of cytosolic cathepsin-B by adding exogenous cathepsin-B to permeabilized pancreatic acini. In this set of experiments, we also incubated permeabilized acini with exogenous trypsin, since trypsin has so far been

considered the major player in cell injury in pancreatitis. We observed that there was dose dependent caspase-3 activation with cathepsin-B but not with trypsin. This convincingly proved that cytosolic cathepsin-B but not trypsin is responsible for acinar cell apoptosis. These observations have been conclusively supported by similar findings from experiments using T7KO and CBKO animals. From these data, the most logical inference is that active trypsin within the co-localized organelles is involved in making the organelles “leaky,” permitting the egress of cathepsin-B into the cytosol, where the released cathepsin-B activated apoptotic pathways. Inhibition of trypsin on the other hand prevented the co-localized organelles from becoming fragile, thereby preventing the release of cathepsin-B into the cytosol. The trypsin that has presumably leaked into the cytosol along with cathepsin-B could not have induced apoptosis since we did not see caspase activation after incubating SLO-permeabilized acinar cells with exogenous trypsin. Requirement of trypsin for release of cathepsin-B also exclude the possibility of its being released from endolysosomal compartment which have been shown to have a role in secretion³⁰.

The next issue that we were interested to examine was how cathepsin-B induced apoptosis in the acinar cells. In mammalian cells, apoptosis occurs via an extrinsic or an intrinsic (or mitochondrial) pathway. Lysosomal disruption has been implicated in initiating the mitochondrial apoptotic pathway involving pro-apoptotic Bcl-2 family member Bid cleavage³¹. Moreover, upon apoptotic stimuli, another Bcl-2 apoptosis-promoting protein Bax undergoes a conformational change and translocates to mitochondria, where it oligomerizes and forms pores that allow the release of cytochrome c³². It has also been shown that in early stages of experimental AP, there is release of cytochrome-c into the cytosol. The released cytochrome-c in turn activates caspase-9, which subsequently activates caspase-3⁹. Caspase-3 then executes the intracellular changes of apoptosis via different downstream mediators. In our experiments also, we found that apoptosis predominantly occurred via the intrinsic or mitochondrial pathway as indicated by active conformational change of Bax, Bid cleavage and an increase in cytosolic cytochrome-c in caerulein-stimulated WT acini. Bax activation, Bid cleavage and caspase-3 activation were fully inhibited in CTSE^{-/-} or T7^{-/-} mice, or WT mice acini pretreated with CA074-me. Moreover, the reduction of cytosolic cytochrome-c after pre-treatment of caerulein-stimulated acini with the cathepsin-B inhibitor CA074-me proved that cathepsin-B triggered acinar cell apoptosis via the intrinsic pathway.

Our results suggest that small amount of Cathepsin-B in the cytosol activates apoptosis and large amount of Cathepsin-B shifts the cell death pathway towards necrosis. In fact the switch of cell death from apoptosis to necrosis based on the amount of cytosolic Cathepsin-B has been described in the literature before¹⁷⁻¹⁹. The mechanism for this kind of switch is not known and is an interesting area of investigation for future studies. It is known though that regulated necrosis induced by multiple triggers involves receptor interacting protein kinase-1 (RIP-1) activating receptor interacting kinase-3 (RIP-3) and forming a complex of RIP1/RIP3 called necrosome complex³³. Activated RIP3 then phosphorylates the downstream substrate that leads to membrane disruption. Intriguingly in our studies we observe increasing RIP-1/RIP-3 complex formation as the cell death during pancreatitis shifts from apoptosis to necrosis. In future studies elucidation of the role of RIP-1/RIP-3 in

the pathogenesis of AP may help decipher the mechanism of selection of apoptosis versus necrotic form of cell death during AP.

In summary, as represented schematically in Figure-7, in this study we have shown for the first time that in experimental pancreatitis, active trypsin within the co-localized organelles makes these organelles fragile and leaky, through which cathepsin-B leaks out into the cytosol. At low level, the released cathepsin-B in the cytosol then induces Bid cleavage and cytochrome c release from mitochondria, leading to apoptosis of acinar cells via the intrinsic pathway. When there is enhanced release of cathepsin-B from these organelles this leads to cell death through necrosis.

Supplementary Material

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Figure-1. Secretagogue induced AP results in release of Cathepsin-B and other lysosomal-enzymes into the cytosol

After appropriate *in vitro* and *in vivo* treatment rat acinar cells were fractionated into cytosol and membrane fraction with Streptolysin O and presence of Cathepsin-B in the cytosol was evaluated. **(A)** Representative western blot demonstrating that supramaximal caerulein stimulation leads to increase in cytosolic cathepsin-B. Actin was used as loading control. **(B)** *In vitro* Supramaximal caerulein and carbachol but not maximal caerulein, carbachol or maximal or supramaximal CCK-JMV 180 leads to increase in cytosolic cathepsin-B activity. **(C)** Supramaximal caerulein stimulation *In vitro* leads to increase in cytosolic activity of the lysosomal enzyme aryl sulfatase. **(D)** Induction of AP in rats with supramaximal caerulein or arginine treatment leads to increase in cytosolic cathepsin-B activity. **(E)** Immunofluorescence evidence of leakage of cathepsin-B into the cytosol in caerulein model of AP (1 dose of 10 µg/kg caerulein with sacrifice after 2 hours) in rats. Cathepsin-B demonstrates punctate green staining in pancreatic acini of control animal while as a result of induction of caerulein pancreatitis cathepsin-B was released into cytosol and gave diffuse staining. **(F)** Cathepsin B immunohistochemistry in pancreatic section from normal donor pancreas or patients with chronic pancreatitis is shown. While cathepsin-B in normal pancreas demonstrates punctate staining, we observed increased number of cells with diffuse cytoplasmic staining in patients with chronic pancreatitis (black arrows). Values of all

enzyme activity are expressed as percent of total (enzymatic activity obtained in the supernatant after lysing the cells with sonication) after normalizing to protein content. All data in (B), (C) and (D) represents mean $\pm$ SEM of triplicates of three individual experiments. * $p < 0.05$ as compared to controls, which were not stimulated with secretagogue or L- arginine.

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Figure-2. Supramaximal caerulein stimulation leads to leakage of cathepsin-B from the co-localized organelles into the cytosol

Supramaximal caerulein stimulation of rat pancreatic acini results in leakage of (A) amylase, (B) trypsin and (C) cathepsin-B into the cytoplasm suggesting that the cytosolic cathepsin-B is arising from organelles containing both lysosomal-enzymes, zymogens and active trypsin i.e. co-localized organelles. Inhibition of co-localization by pre-treatment with the PI-3 kinase inhibitors Wortmannin and Ly492004, leads to significant reduction in cytosolic amylase, trypsin and cathepsin-B activity again suggesting that co-localized organelles are the source of the cytosolic Cathepsin-B. (D) HSP70 induction by prior thermal stress or sodium arsenite prevents cytosolic cathepsin-B increase in response to supramaximal caerulein. (E) Attenuation of cytosolic calcium reduces cytosolic cathepsin-B activity

following supramaximal caerulein. **(F)** Cytosolic cathepsin-B activity normalized to LDH is increased in mice pancreatic acini treated with supramaximal caerulein. However, supramaximal caerulein is unable to cause release of Cathepsin-B into the cytosol of trypsinogen-7 (T7KO) mice. Values of enzyme activity in A-E are expressed as percent of total (enzymatic activity obtained in the supernatant after lysing the cells with sonication) after normalizing protein content. Cathepsin-B activity is normalized to LDH content in F. All data represents mean $\pm$ SEM of triplicates of three individual experiments. * $p < 0.05$ as compared to caerulein only stimulation.

Figure-3. Supramaximal caerulein *in vitro* and *in vivo* and L- arginine *in vivo* leads to apoptosis in rat pancreatic acini

(A) Supramaximal caerulein stimulation of rat acini *in vitro* leads to caspase-3 activation. Caspase-3 activity was measured using Caspase-Glo® 3/7 Assay from promega. Supramaximal caerulein stimulation of acini also leads to increase in (B) green fluorescence (yellow arrow) and (C) Apo Trace fluorescence after dye extraction suggesting occurrence of apoptosis. (D) *In vivo* induction of AP by either supramaximal caerulein or L-arginine leads to increase in caspase-3 activation. Caerulein induced acinar cell apoptosis, as measured by (E) Apo Trace fluorescence or (F) caspase-3 activity, is decreased in acini pre-treated with either cathepsin-B inhibitor CA074-me or trypsin inhibitor benzamidine. Values are expressed as percent of control and normalized to per mg protein and all data represents

mean $\pm$ SEM of triplicates of three individual experiments. * $p < 0.05$ as compared to controls, which were not stimulated with caerulein or L-arginine.

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Figure-4. Cytosolic cathepsin-B but not trypsin is responsible for acinar cell apoptosis

Introduction of exogenous Cathepsin-B but not trypsin into SLO permeabilized normal rat pancreatic acinar cells leads to caspase-3 activation. Caspase-3 activity was noticed over a wide range of doses of cathepsin-B while no activity was seen with any given dose of trypsin. Values are expressed as percent of total and normalized to per mg protein. All data represents mean $\pm$ SEM of triplicates of three individual experiments.

Figure-5. Cathepsin-B and trypsin are required for activation of caspase 3 and acinar cell death (A) Supramaximal caerulein stimulation reduced WT pancreatic acinar cell viability, whereas CBKO and T7KO acini were protected against caerulein induced cell death. Pretreatment of pancreatic acini with cathepsin-B inhibitor CA074me (10 μ M for 15 minutes) also protected acini against cell death caused by supramaximal caerulein. Acini were stimulated using either maximal or supramaximal concentrations of caerulein for 3hrs. Cell viability was quantitated using MultiTox-Fluor Multiplex Cytotoxicity Assay from Promega. (B) Supramaximal caerulein activated caspase-3 in WT acini, but CA074-me pretreatment protected WT acini against caspase-3 activation. In CBKO acini which lack cathepsin-B or T7KO acini which do not show trypsin activation no caspase-3 activity was detected in acinar cells after supramaximal stimulation with caerulein. (C) Exogenous cathepsin-B activates caspase-3 in permeabilized WT or CTSB^{-/-} pancreatic acinar cells. Supramaximal caerulein stimulation does not augment caspase-3 activation over and above that induced by addition of exogenous cathepsin-B to permeabilized WT and CTSB^{-/-} mice pancreatic acini. (D) Non-permeable CA074 treatment blocks exogenous cathepsin-B-induced caspase-3 activation in permeabilized WT mice pancreatic acinar cells. WT mice pancreatic acini were either mock- or pre-incubated for 20 min with 10 μ M permeable CA074-me, followed by treatments for 1 h with 10⁻⁷ M caerulein after SLO permeabilization. The cells were subsequently mock-incubated or incubated with CA074 for

20 min followed by exposure to exogenous cathepsin-B for 3hrs. Caspase-3 activity was then quantified and normalized to DNA content.

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Figure-6. Cathepsin-B activates the mitochondrial apoptosis pathway. Excessive amount of cytosolic cathepsin-B causes necrotic cell death

(A) Caerulein hyperstimulation leads to Bid cleavage in WT but not in CTSE^{-/-} mice. WT or CTSE^{-/-} mice were injected intraperitoneally with caerulein (50 µg/kg/injection, for 3 hourly injections, animals were sacrificed 30 min or 60 min after injection and pancreatic tissues probed with Bid and GAPDH antibodies. (B) Quantification of bid cleavage from three independent experiments is shown. (C) **Cytosolic cathepsin-B induces Bax activation in response to supramaximal caerulein stimulation.** WT and CTSE^{-/-} mice pancreatic acini were untreated or treated with 10^{-10} M, or 10^{-7} M caerulein for 3h. Cell lysates (400 µg) were immunoprecipitated with 0.2µg of the mouse anti-Bax antibody (BD Pharmingen), and the immunoprecipitates were examined by immunoblot analysis with active Bax monoclonal antibody 6A7. (D) Caerulein stimulation of pancreatic acinar cells caused release of cytochrome c into the cytosol of WT mice. This caerulein induced Cathepsin-B release was prevented by pre-treatment with Cathepsin-B inhibitor CA074-me. GAPDH was used as loading control for cytosolic fraction and COX IV for mitochondrial fraction. Cytosolic and

membrane fractions of the acini were separated after permeabilization with SLO. Role of cathepsin-B induced cytochrome C was further evaluated by evaluating effect of addition of extrinsic cathepsin-B to the permeabilized acini with and without neutralizing anti-cytochrome c antibody. While addition of extrinsic cathepsin-B to the permeabilized acini in presence of control isotype specific antibody causes caspase-3 activation, neutralization of the cytochrome c prevents cathepsin-B induced caspase 3 activation. **(E)** LLOMe leads to release of cathepsin-B into the cytosol of acinar cells in a dose dependent fashion. At lower doses there is activation of caspase-3 which decreases at higher dose of LLOMe with corresponding increase in LDH release suggesting shift from apoptosis to necrosis. **(F)** While higher dose of LLOMe is able to induce necrosis in acini from wild type mice, no necrosis was observed in cathepsin-B knockout mice when treated with LLOMe. Results are expressed as fold change compared with untreated controls. Each value is the mean of triplicate samples. Representative data of three independent experiments are shown.

Figure 7.
Schematic representation of the new paradigm of cell death in experimental AP.