Cell Death in Pancreatitis
CASPASES PROTECT FROM NECROTIZING PANCREATITIS*

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Mechanisms of cell death in pancreatitis remain unknown. Parenchymal necrosis is a major complication of pancreatitis; also, the severity of experimental pancreatitis correlates directly with necrosis and inversely with apoptosis. Thus, shifting death responses from necrosis to apoptosis may have a therapeutic value. To determine cell death pathways in pancreatitis and the possibility of necrosis/apoptosis switch, we utilized the differences between the rat model of cerulein pancreatitis, with relatively high apoptosis and low necrosis, and the mouse model, with little apoptosis and high necrosis. We found that caspases were greatly activated during cerulein pancreatitis in the rat but not mouse. Endogenous caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP) underwent complete degradation in the rat but remained intact in the mouse model. Furthermore, XIAP inhibition with embelin triggered caspase activation in the mouse model, implicating XIAP in caspase blockade in pancreatitis. Caspase inhibitors decreased apoptosis and markedly stimulated necrosis in the rat model, worsening pancreatitis parameters. Conversely, caspase induction with embelin stimulated apoptosis and decreased necrosis in mouse model. Thus, caspases not only mediate apoptosis but also protect from necrosis in pancreatitis. One protective mechanism is through degradation of receptor-interacting protein (RIP), a key mediator of “programmed” necrosis. We found that RIP was cleaved (i.e. inactivated) in the rat but not the mouse model. Caspase inhibition restored RIP levels; conversely, caspase induction with embelin triggered RIP cleavage. Our results indicate key roles for caspases, XIAP, and RIP in the regulation of cell death in pancreatitis. Manipulating these signals to change the pattern of death responses presents a therapeutic strategy for treatment of pancreatitis.

Acute pancreatitis is an inflammatory disorder of exocrine pancreas, which carries considerable morbidity and mortality, and the pathophysiology of which remains obscure (1). During the past decade, significant progress has been achieved in our understanding of the inflammatory response in pancreatitis (2–5). By contrast, very little is known about the mechanisms mediating another major pathologic response in pancreatitis, the parenchymal cell death.

In experimental models of acute pancreatitis, acinar cells have been shown to die through both necrosis and apoptosis (6, 7). The apoptosis/necrosis ratio varies in different experimental models of pancreatitis. Of note, the severity of experimental pancreatitis directly correlates with the extent of necrosis and inversely with that of apoptosis (6–12). Mechanisms underlying these differences are not known.

Apoptosis and necrosis are two main types of cell death (13–18). Morphologically, apoptosis is manifested by cell shrinkage and chromatin condensation, whereas necrosis is characterized by swelling of the cell and its organelles and rupture of the plasma membrane. Biochemical hallmarks of apoptosis, such as activation of specific cysteine proteases, the caspases, and internucleosomal DNA fragmentation, are usually absent in necrotic cells. Apoptosis preserves the structural integrity of the plasma membrane, whereas the necrotic cell releases its constituents, which damage neighboring cells and promote inflammatory infiltration in the organ. Therefore, necrotic death is “deadlier” to the organism than apoptotic death (13–15).

There are two distinct pathways of apoptosis (19–21). The extrinsic pathway is initiated by receptor-induced activation of the initiator caspase-8 (or caspase-10) followed by activation of effector caspases such as caspase-3. This pathway is typically triggered by “death receptors”, e.g. tumor necrosis factor receptor or Fas. In the intrinsic pathway, a critical event is permeabilization of the mitochondrial outer membrane, resulting in the release of pro-apoptotic factors such as cytochrome c. Once released, cytochrome c forms a complex with Apaf-1 and procaspase-9, resulting in caspase-9 activation. Caspase-9 further cleaves and activates the effectors caspases, (e.g. caspase-3) leading to subsequent degradation of cellular constituents.

Inhibitor of apoptosis proteins (IAPs)2 are an important class of endogenous proteins that negatively regulate caspase activation (22–24). The X-linked IAP (XIAP) is the most potent among the eight mammalian IAPs and inhibits the mitochondria-driven caspases-9, -3, and -7 (23, 25).

In contrast with other disease states, for example, myocardial infarction (26, 27) or ischemic renal failure (28), there is very little known about the signaling mechanisms of apoptosis in pancreatitis. We have recently shown (29) that the apoptotic “machinery” is present in isolated rat pancreatic acinar cells and can be activated by supramaximal doses of cholecystokinin-8 (CCK-8), which cause pancreatitis-like changes in acinar cells. In particular, CCK-8 induced mitochondrial cytochrome c release and activation of caspases-9, -3, and -8.

In contrast with apoptosis, the signaling mechanisms mediating

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2The abbreviations used are: IAP, inhibitor of apoptosis protein; FLIP, FLICE-inhibitory protein; RIP, receptor-interacting protein; Q-VD-OPH, Q-Val-Asp(nomethylated)-Oph; XIAP, X-linked inhibitor of apoptosis protein; Z-D-DCB, Z-Asp-2,6-dichlorobenzoyloxymethylketone; Z, benzoyloxy carbonyl, fmk, fluoromethyl ketone; CCK-8, cholecystokinin-8; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; AMC, 7-amino-4-methylcoumarin; ERK, extracellular signal-regulated kinase; E3, ubiquitin-protein isopeptide ligase.

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necrotic cell death are in general poorly understood. Recent findings indicate that there are two types of necrosis (15–17). Accidental necrosis is an unregulated process triggered by severe cellular stress that is often characterized by depletion of cellular ATP. It is seen as a passive explosion of a cell overwhelmed by ion fluxes. By contrast, so-called programmed necrosis (or necrosis-like programmed cell death) is mediated by a coordinated series of signaling events that can be triggered via death receptors. For example, depending on conditions the same tumor necrosis factor receptor could induce either apoptosis or necrosis (30, 31). The only so far proven mediator of programmed necrosis is the receptor-interacting protein kinase (RIP). RIP deficiency rescues cells from tumor necrosis factor–induced programmed necrosis (32–34). The mechanisms of RIP action, as well as its targets, are poorly understood (15–17, 30).

Acinar cell necrosis, and in particular, recurrent necrosis, is one of the most serious complications of acute pancreatitis (1, 35, 36). Based on the above-described observations that milder forms of experimental pancreatitis are associated with more apoptosis and the relatively severe forms, with more necrosis, it has been hypothesized (6–12) that switching from the necrotic pattern of cell death to apoptosis could be beneficial in the treatment of acute pancreatitis.

In the present study we investigated the mechanisms of apoptosis and necrosis in pancreatitis and explored the possibility of necrosis/apoptosis switch through manipulating these signaling mechanisms. For this purpose, we utilized the differences between two related rodent models of acute pancreatitis. Pancreatitis induced in rats by supramaximally stimulating doses of the CCK-8 analog, cerulein, is a mild form of the disease characterized by relatively high extent of apoptosis and low necrosis (7, 9). By contrast, in mice the same cerulein treatment results in a more severe disease with significant necrosis and very little apoptosis (7). Both are the most commonly used and well characterized in vivo models of acute pancreatitis (37, 38).

We found drastic differences in death-signaling mechanisms, namely, caspase activation, and XIAP and RIP degradation, between the rat and mouse models of cerulein pancreatitis. By manipulating these mechanisms using pharmacologic inhibitors, we were able to shift the necrosis/apoptosis ratio, making the two models more like each other. The results identify several critical mediators of acinar cell death that may represent targets for therapeutic interventions to attenuate cell-death responses of acute pancreatitis.

**EXPERIMENTAL PROCEDURES**

**Experimental Pancreatitis**—Cerulein pancreatitis was induced in male (200–250 g) Sprague-Dawley rats and male (25–30 g) Swiss Webster CD-1 mice by up to seven hourly intraperitoneal injections of 50 µg/kg cerulein. Control animals received similar injections of physiologic saline. Caspase inhibitors Q-VD-OPH (25 mg/kg) and Z-D-DCB (10 mg/kg), or vehicle (Me2SO), were applied in rats as a single intravenous injection 30 min before the start of cerulein treatment. XIAP inhibitor embelin (20 mg/kg), or vehicle, were applied in mice as one daily subcutaneous injection for 5 consecutive days; treatment with cerulein started 30 min after the last embelin injection. In the cerulein models, animals were sacrificed at 30 min, 2, 4, and 7 h after the first injection. Arginine pancreatitis was induced in rats by two hourly intraperitoneal injections of 2.5 g/kg L-arginine; controls received similar injections of saline. In this model, rats were sacrificed 24 h after the first injection. Animals were euthanized by CO2–induced asphyxiation, and the blood and pancreas were harvested for measurements.

**Serum Amylase and Lipase Measurements**—Serum amylase and lipase levels were measured in a Hitachi 707 analyzer (Antech Diagnostics, Irvine, CA).

**Quantification of Apoptosis**—Apoptosis was quantified on pancreatic tissue sections stained with Hoechst 33258 to visualize nuclear chromatin morphology or with TUNEL assay to measure DNA breaks, as we described previously (3, 6, 9, 39). Tissue was fixed in 4% buffered formaldehyde and embedded in paraffin, and 6 µm-thick sections were adhered to glass slides. Sections were deparaffinized by washing in Hemo-De and hydrated by transferring through graded ethanol. The sections were stained with 8 µg/ml Hoechst 33258 and examined by fluorescence microscopy. Nuclei with condensed or fragmented chromatin were considered apoptotic (12, 39). In TUNEL assay (6), tissue sections were stained for breaks in DNA using terminal deoxynucleotidyl transferase and fluorescein isothiocyanate–labeled dUTP according to the manufacturer’s protocol (Promega, Madison, WI). For these and other quantifications of histologic measurements, a total of at least 1000 acinar cells was counted on pancreatic tissue sections from each animal.

**Quantification of Necrosis**—Quantification of necrosis was performed on pancreatic tissue sections stained with H&E. Cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into interstitium were considered necrotic.

**Quantification of Inflammatory Infiltration**—Quantification of inflammatory infiltration was performed on pancreatic tissue sections stained with H&E.

**Isolation of Pancreatic Acini**—Isolation of pancreatic acini from rats or mice was performed using a collagenase digestion procedure as we described previously (3, 29, 39). Dispersed pancreatic acini were then incubated at 37 °C in 199 medium (Invitrogen) in the presence or absence of 100 nM CCK-8.

**Caspase Activities**—Caspase activities were measured using a fluorogenic assay as we described previously (29, 40). Pancreatic tissue or acinar cell samples were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Igepal CA-630, and 0.5 mM EDTA, centrifuged for 15 min at 16,000 × g, and the supernatants were collected. Proteolytic reactions were carried out at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, and 10 mM dithiothreitol, using substrates specific for caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), or caspase-9 (Ac-LEHD-AMC).

Cleavage of these substrates releases 7-amino-4-methylcoumarin (AMC), which emits fluorescent signal with excitation at 380 nm and emission at 440 nm. Fluorescence was calibrated using a standard curve for AMC. The data are expressed as moles of AMC/mg of protein/min.

**Preparation of Tissue and Cell Lysates for Western Blot Analysis**—Portions of frozen tissue were homogenized on ice in radioimmune precipitation assay buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and the protease inhibitor mixture containing pepstatin, leupeptin, chymostatin, antipain, and aprotinin (5 µg/ml of each), rotated for 20 min at 4 °C, and centrifuged at 4 °C for 15 min at 16,000 × g. The supernatants were collected and stored at −80 °C. Dispersed pancreatic acini were washed twice with ice-cold phosphate-buffered saline, resuspended in radioimmune precipitation assay buffer, and processed as described above for tissue samples. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

**Preparation of Membrane and Cytosolic Fractions**—Pancreatic tissue or acinar cell samples were homogenized in a buffer containing 250 mM sucrose, 20 mM HEPES-KOH (pH 7.0), 10 mM KCl, 1 mM EGTA, 2 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor mixture in a glass Dounce homogenizer (80 strokes). Nuclei were removed by centrifugation at 1,000 × g.
for 10 min at 4 °C. The supernatant was centrifuged for 1 h at 100,000 × g, and both the pellet (mitochondria-enriched membrane fraction) and supernatant (cytosolic fraction) were collected separately and used for Western blotting.

Western Blot Analysis—was performed on total tissue and cell lysates, or on the membrane and cytosolic fractions, as we described previously (3, 29, 39, 40). Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by 1-h incubation of the membranes in 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.5). The blots were then incubated for 2 h or overnight with primary antibodies in the antibody buffer containing 1% (w/v) nonfat dry milk in TTBS (0.05% (v/v) Tween 20 in Tris-buffered saline), washed three times with TTBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. The blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce). Band intensities in the immunoblots were quantified by densitometry.

Reduction/Alkylation—This was done according to a previous study (41), with minor modifications. Briefly, proteins in tissue lysate were reduced in 0.1 M Tris-HCl (pH 9.0) containing 8 M urea and 0.1 M dithiothreitol, and incubated at 37 °C for 1 h. Alkylation of reduced proteins was achieved by incubation for 30 min with 0.3 M iodoacetamide at room temperature in the dark. The samples were then separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot analysis with antibody against cytochrome c.

Antibodies and Reagents—Antibodies against XIAP and p44/42 mitogen-activated protein kinase (Erk1/2) were from Cell Signaling (Beverly, MA); caspase-3, caspase-8, and FLIP, from Santa Cruz Biotechnology (Santa Cruz, CA); cytochrome c, RIP, and Pyk2, from BD Biosciences (San Diego, CA); COX IV, from Molecular Probes (Eugene, OR); and caspase-9, from Stressgen (San Diego, CA). CCK-8 was from American Peptide (Sunnyvale, CA); cerulein was from Peninsula Laboratories (Belmont, CA). Caspase fluorogenic substrates Ac-IETD-AMC, Ac-DEVD-AMC, and Ac-LEHD-AMC, and the XIAP inhibitor embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) were from Biomol (Plymouth Meeting, PA). Caspase inhibitors Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-D-DCB) and Q-Val -Asp(non-O-methylated)-OPh (Q-VD-OPh) were from ALEXIS Biochemicals (San Diego, CA) and Enzyme Systems Products (Livermore, CA), respectively. Other reagents were from Sigma.

RESULTS

In rat and mouse cerulein pancreatitis, we measured time-dependent changes in the extent of apoptosis and necrosis in pancreas (Fig. 1). In the rat model, the extent of apoptosis was unchanged at 30 min after the start of cerulein treatment and increased ~33-fold at 4 h and ~16-fold at 7 h. The decrease in apoptosis at 7 h could be due to an increased clearance of apoptotic cells by inflammatory cells, the number of which in pancreas increases with time. Compared with the rat model, there was much less apoptosis in mouse cerulein pancreatitis, and the increase in apoptosis was minimal. Necrosis time dependently increased in both models of cerulein pancreatitis. In the rat model, the increase in necrosis was only detected at 4 and 7 h, whereas in the mouse model necrosis was already evident at 30 min. At all time points, necrosis in the rat was several times less than in the mouse model. Fig. 1 illustrates the reciprocal pattern of cell-death responses in the rat versus mouse cerulein pancreatitis. For example, at 7 h the extent of apoptosis in the rat model was ~8-fold higher and that of necrosis, ~5-fold lower than in the mouse.

Apoptotic Signaling Pathways in Cerulein Pancreatitis

Caspases Are Greatly Activated in the Rat but Not Mouse Cerulein Pancreatitis—We measured the effects of pancreatitis on both the effector caspase-3 and initiator caspases-9 and -8 (Fig. 2). In rat pancreas, caspase-3 antibody recognized only one pro-caspase-3 band (~34 kDa), whereas in the mouse pancreas this antibody detected two bands of ~34 and ~37 kDa (Fig. 2A, left panel), which may reflect different phosphorylation states of procaspase-3 (18, 42). In the course of cerulein pancreatitis in the rat, caspase-3 underwent time-dependent processing manifest by a decrease in the 34-kDa proform and the appearance of the cleaved ~17-kDa product, which represents the active form of caspase-3 (29, 43). Caspase-3 processing was first evident at 30 min. In contrast, we detected no processing of caspase-3 in mouse cerulein pancreatitis (Fig. 2A, right panel). In parallel with the observed processing of procaspase-3, pancreatic caspase-3 activity (measured with a fluorogenic assay) greatly increased in rat cerulein pancreatitis compared with untreated or control, saline-injected animals (Fig. 2B). This increase leveled off at ~15-fold between 2 and 4 h of cerulein treatment. No increase in caspase-3 activity was detected in the mouse model (Fig. 2B).

Caspase-9 processing (29, 43, 44) was detected in rat cerulein pancreatitis by time-dependent accumulation of the ~40-kDa cleaved product (already evident at 30 min) and concomitant decrease in the ~50-kDa proform (Fig. 2A, left panel). In contrast, no processing of pancreatic caspase-9 was detected in the mouse model. Measurements of caspase-9 activity paralleled those of its processing: caspase-9 activity increased up to ~10-fold in rat cerulein pancreatitis whereas no activation of caspase-9 was detected in the mouse model (Fig. 2B).

In both rat and mouse pancreas, caspase-8 antibody recognized two bands likely corresponding to the 2 main procaspase-8 isoforms (45). In rat cerulein pancreatitis caspase-8 processing was already manifest at 30
min, resulting in accumulation of the ~50-kDa cleaved product (the intermediate form). We did not detect the completely processed caspase-8 (the p16 product (45)). It has been shown (46) that caspase-8 can be activated not only through its cleavage to the p16 form but also through oligomerization of the intermediate products. Despite incomplete processing, the activity of caspase-8 greatly (up to 20-fold) increased in rat cerulein pancreatitis. As with the other caspases, we detected neither processing nor activation of caspase-8 in mouse cerulein pancreatitis (Fig. 2).

The results in Fig. 2 show that both initiator (caspases-8 and -9) and effector (caspase-3) caspases are greatly activated in rat cerulein pancreatitis. In contrast, neither caspase processing nor their increased activity was detected in the mouse model.

Activation of both initiator and effector caspases occurred early in rat cerulein pancreatitis, with processing and increased activity being evident at 30 min of cerulein treatment. The kinetics of activation was different for the initiator and effector caspases: the activities of caspase-9 and -8 increased almost linearly during the whole period of observation, whereas the increase in caspase-3 activity leveled off at 4 h.

To determine whether the differences in caspase activation found in the rat and mouse models of cerulein pancreatitis occur at the level of acinar cells, we compared the effects of CCK-8 on caspase activities in acinar cells isolated from rat and mouse pancreas. As we showed previously (29), in rat pancreatic acinar cells supramaximal CCK-8 caused a rapid and pronounced activation of all 3 caspases (Fig. 3). By contrast, in mouse pancreatic acinar cells supramaximal CCK-8 caused a rapid and pronounced activation of all 3 caspases (Fig. 3). By contrast, in mouse pancreatic acinar cells supramaximal CCK-8 caused a rapid and pronounced activation of all 3 caspases (Fig. 3). These results indicate that the “caspase block” in mouse cerulein pancreatitis occurs in acinar cells.

Of note, the procedure of acinar cell isolation from the pancreas induces various stresses in isolated acini (47). In particular, basal caspase
activities are greater in isolated rat acinar cells than in pancreatic tissue from untreated or saline-treated rats (29).

Cytochrome c Release Occurs in Both Models of Cerulein Pancreatitis—One possible reason for the observed differences in caspase activation between the rat and mouse models could be a blockade of mitochondrial cytochrome c release upstream of caspase-9 in mouse cerulein pancreatitis. We found, however, that pancreatic cytochrome c release occurred in both models (Fig. 4A), as was manifested by time-dependent decrease in the membrane 14-kDa cytochrome c on Western blot performed on membrane fractions from pancreatic tissue. Concomitantly, cytochrome c immunoreactivity increased in the cytosolic fractions from pancreatic tissue in both models (Fig. 4A). Surprisingly, in the rat model we observed a time-dependent decrease in the cytosolic 14-kDa cytochrome c band, which was accompanied by accumulation of a ~30-kDa band. This band was detected by both monoclonal (BD Bioscience, Fig. 4A) and polyclonal (Santa Cruz Biotechnology, not shown) cytochrome c antibodies; further, preincubation of the polyclonal antibody with blocking peptide prevented recognition of both 14- and 30-kDa bands, indicating that both bands correspond to cytochrome c (data not shown).

Unlike the rat model, in mouse cerulein pancreatitis the intensity of the cytosolic 14-kDa cytochrome c band only increased with time (Fig. 4A, right panel). The ~30-kDa band was also present in the cytosolic cytochrome c immunoblots from mouse pancreatitis tissue; its intensity increased much more slowly than in the rat model and was only prominent at 7 h (Fig. 4A).

The nature of the 30-kDa form of cytosolic cytochrome c remains to be determined. One possible explanation is that this form represents the cytochrome c dimer. It has been reported that cytochrome c could form oligomers and that its oligomerization is potentiated by the neuronal protein α-synuclein (48). An-synuclein derived from nerves could be present in pancreatic tissue homogenates and promote cytochrome c oligomerization. The more cytochrome c released from the mitochondria during the course of pancreatitis, the greater the extent of its oligomerization in the cytosol and thus the intensity of the 30-kDa band. To demonstrate that the 30-kDa cytochrome c band was associated with oligomerization, protein samples were subjected to a reduction/alkylation procedure (41) prior to SDS-PAGE. The reduction/alkylation procedure abrogated the 30-kDa band (Fig. 4D), indicating that this band results from complex formation involving disulfide linkages.

The presence of the 30-kDa band complicated the comparison of cytosolic cytochrome c levels between the rat and mouse models. We estimated the changes in cytosolic cytochrome c levels by measuring the sum of the intensities of both 14- and 30-kDa bands. The densitometric quantification (Fig. 4B) showed that the total cytochrome c accumulation in the cytosol was more rapid in the rat model, reaching the maximal level by 2–4 h of cerulein treatment. However, by 7 h the increases in total cytosolic cytochrome c were of similar magnitude in both models of cerulein pancreatitis.

We further measured cytochrome c release in isolated pancreatic acinar cells. In rat acinar cells, as we showed previously (29), supramaximal CCK-8 induced a decrease in membrane cytochrome c with a concomitant increase in the cytosolic 14-kDa cytochrome c (Fig. 4C). A similar decrease in the membrane cytochrome c with concomitant increase in the cytosolic cytochrome c was observed in isolated mouse pancreatic acinar cells treated with CCK-8 (Fig. 4C). Of note, we only detected the 14-kDa cytosolic cytochrome c, but not the 30-kDa band, in both rat and mouse acinar cells. Whether the absence of the 30-kDa cytosolic cytochrome c band in isolated pancreatic acinar cells is due to the absence of the nerve-derived α-synuclein (or other factors facilitating cytochrome c oligomerization) remains to be determined.

The results in Figs. 2–4 show that cytochrome c release from mitochondria occurs in both models of cerulein pancreatitis; however, in the mouse model it does not translate into caspase activation. This indicates that the caspase block we found in the mouse model is not due to a blockade of cytochrome c release. Therefore, we next investigated the effect of pancreatitis on endogenous protein inhibitors of caspases.

XIAP Mediates the Caspase Block in Mouse Cerulein Pancreatitis—We first evaluated the effect of pancreatitis on c-FLIP, a cytosolic protein with homology to caspase-8 that acts as a dominant-negative inhibitor of caspase-8 activation (49). There are two splice isoforms of c-FLIP, one of which, the c-FLIPL (short isoform), completely blocks caspase-8 processing, whereas the other, c-FLIPs (long isoform), allows partial processing of caspase-8 (49–51). We found that both c-FLIPL and c-FLIPs were markedly up-regulated in both rat and mouse cerulein pancreatitis (Fig. 5A). The up-regulation of c-FLIP isoforms occurred faster in the rat model, but the magnitude of the response was comparable in both models. Thus, changes in c-FLIP cannot account for the differences in caspase activation between rat and mouse models of cerulein pancreatitis (if anything, c-FLIP up-regulation was greater in the rat).

We next asked whether the blockade of the mitochondria-driven caspase-9 (and subsequently, caspase-3) activation in the mouse model occurs downstream of cytochrome c release, at the level of IAPs. Western blot analysis showed the presence of XIAP, a key member of the IAP family (22–24), in both rat and mouse pancreas (Fig. 5). However, the effect of cerulein pancreatitis on XIAP was drastically different between the rat and mouse models. XIAP was rapidly and fully degraded in the rat model but its level remained unchanged in mouse cerulein pancreatitis.
Necrosis/Apoptosis Switch in Experimental Acute Pancreatitis

To determine the role of caspases in cell-death responses of pancreatitis, we first measured the effects of caspase inhibition on apoptosis and necrosis in the rat model of cerulein pancreatitis in which caspases were greatly activated (Fig. 2). For this purpose we used broad-spectrum peptide caspase inhibitors Z-D-DCB (59) and Q-VD-OPH (28, 60). We did not apply another broad-spectrum caspase inhibitor, the commonly used Z-VAD-fmk (61), because in experiments on isolated acinar cells we found that Z-VAD-fmk, in addition to caspases, also inhibited cathepsin B activity (data not shown). By contrast, neither Z-D-DCB nor Q-VD-OPH affected cathepsin B in acinar cells (not shown).

Q-VD-OPH completely, and Z-D-DCB partially, inhibited activation of caspase-3 (Fig. 6A) and caspases-9 and -8 (data not shown) in rat cerulein pancreatitis. Increasing Z-D-DCB concentration did not further increase the extent of caspase inhibition (not shown). Both inhibitors decreased pancreatic apoptosis in rat cerulein pancreatitis as measured by both Hoechst 33258 and TUNEL staining (Fig. 6B). Importantly, although Q-VD-OPH completely prevented caspase activation in rat cerulein pancreatitis, it only inhibited apoptosis by $\sim$50% (Fig. 6, A and B). These results...
indicate an equally important contribution of caspase-dependent and -in-dependent pathways to apoptosis in this model of pancreatitis. Caspase inhibition increased necrosis in rat cerulein pancreatitis 3-fold (Figs. 6C and 7A). It also worsened other parameters of pancreatitis, namely, the increases in serum amylase and lipase (Fig. 7, B and C) and inflammatory cell infiltration in the pancreas (Fig. 7D).

To further elucidate the role of caspases in the regulation of cell-death responses, we measured the effect of the XIAP inhibitor embelin on apoptosis and necrosis in the mouse model of cerulein pancreatitis, in which caspases are silent. As shown in Fig. 5, in this model embelin induced activation of caspases-9, -3, and -8. Embelin caused a 3-fold increase in apoptosis (Fig. 8A); furthermore, caspase activation by embelin resulted in a significant decrease in necrosis and normalization of pancreatic histology in mouse cerulein pancreatitis (Fig. 8, B and C). The results in Figs. 6–8 show that caspases not only mediate apoptosis but also protect from necrosis in cerulein pancreatitis.
bition stimulated necrosis, whereas their activation inhibited necrosis. Fig. 9 summarizes the data on the effects of modulating caspase activity on necrosis/apoptosis ratio in the two models of cerulein pancreatitis.

At the indicated time points, the necrosis/apoptosis ratio is ~120-fold greater in the mouse than in the rat model. Caspase inhibition increased this ratio in rat cerulein pancreatitis (Fig. 6); conversely, caspase induction with embelin decreased the necrosis/apoptosis ratio in mouse cerulein pancreatitis (Fig. 8) by stimulating apoptosis and inhibiting necrosis. Thus, manipulating caspase activities caused necrosis/apoptosis switch and made the patterns of cell-death responses in the two models more similar (Fig. 9).

RIP Degradation by Caspases Correlates with Low Necrosis in Experimental Pancreatitis

Compared with apoptosis, the signaling pathways mediating necrosis are much less understood (15–17). RIP kinase has recently emerged as a
key mediator of necrosis-like programmed cell death (15–17, 32–34). We found that the effect of cerulein pancreatitis on RIP was drastically different between the rat and mouse models (Fig. 10A). In rat cerulein pancreatitis, RIP underwent rapid and full cleavage to a ∼47-kDa product. In contrast, there was very little cleavage of RIP in the mouse model (Fig. 10A). To test that RIP cleavage is not species-specific, we measured that RIP was not cleaved in rat arginine pancreatitis (Fig. 10B); thus, RIP behaves differently in the two rat models of acute pancreatitis.

RIP can be cleaved (and thus inactivated) by caspases-8 and -3 (62–64). To determine whether RIP cleavage observed in cerulein pancreatitis is mediated by caspases, we measured the effects of caspase inhibitors and embelin on RIP. Caspase inhibition with Q-VD-OPH markedly decreased RIP cleavage in rat cerulein pancreatitis (Fig. 10C). Conversely, stimulation of caspase activity by embelin induced RIP cleavage in mouse cerulein pancreatitis (Fig. 10D). These results indicate that caspases mediate RIP degradation in cerulein pancreatitis.

Thus, RIP cleavage directly correlates with caspase activation and apoptosis, and inversely, with the extent of necrosis in the experimental models of acute pancreatitis we applied. Such correlations suggest that RIP cleavage (i.e., inactivation) could be one mechanism through which caspases inhibit necrosis.

**DISCUSSION**

Inflammation and parenchymal cell death are hallmarks of pancreatitis (1, 2). In the past decade, significant progress has been achieved in understanding the mechanisms of the inflammatory response of pancreatitis (2–4, 9, 39, 65, 66). In contrast, the mechanisms of cell-death responses of pancreatitis remain largely unexplored. To determine the mechanisms mediating necrosis and apoptosis in pancreatitis, in the present study we analyzed death-signaling pathways in rat and mouse cerulein pancreatitis, the most commonly used and well characterized models of acute pancreatitis (37, 38). Cerulein, an analog of CCK-8, interacts with CCK receptors on the pancreatic acinar cell (38, 67). Both the rat and mouse models display key responses of acute pancreatitis such as the dysregulation of digestive enzymes’ secretion (i.e., increased levels of serum amylase and lipase); premature, intrapancreatic trypsinogen activation; activation of transcription factor NF-κB resulting in up-regulation of pro-inflammatory cytokines and chemokines; inflammatory cell infiltration; and parenchymal cell death (2, 3, 5, 9, 38, 66). However, the patterns of death responses differ in these two models of acute pancreatitis induced by the same treatment. Rat cerulein pancreatitis is a mild disease characterized by low necrosis and relatively high apoptosis, whereas the mouse cerulein model is a more severe disease with high necrosis and very little apoptosis. Indeed, our data show that the necrosis/apoptosis ratio in the mouse model is ∼120-fold higher than in the rat model. (It is worth noting that actual rates of apoptosis could be higher, because the remnants of cells dying through apoptosis (i.e., apoptotic bodies) are rapidly phagocytosed (68).) Indeed, we showed (9) that depletion of the inflammatory cells increased apoptosis in the rat cerulein pancreatitis up to 17%. By contrast, phagocytosis of necrotic cell’s remnants is much less efficient (69).

We utilized the differences in cell-death responses between the two rodent models of cerulein pancreatitis to elucidate the mechanisms mediating necrosis and apoptosis in pancreatitis and to explore the possibility of manipulating the death responses for therapeutic purposes. We found that both the effector caspase-3 and initiator caspases-9 and -8 are rapidly and greatly activated in rat cerulein pancreatitis. By contrast, there was no caspase activation in the mouse model. Similarly, in vitro supramaximal CCK-8 induced caspase activation in acinar cells isolated from rat but not mouse pancreas. Thus, one difference in cell-death responses between the rat and mouse models of cerulein pancreatitis is the caspase block in mouse pancreatic acinar cells.

Cytochrome c release occurred in both models of cerulein pancreatitis. This indicates that mitochondrial damage is induced in experimental acute pancreatitis, suggesting a role for the mitochondrial pathway in
Inhibition of caspase-9 (54), it induced activation of all three caspases (caspase-3) by XIAP and is currently in phase I of clinical trials for cancer treatment (71). XIAP inhibitor embelin (54), was shown to prevent the inhibition of caspase-9 (but not caspase-8) on activation of XIAP with embelin could be through cleavage (i.e., inactivation) of RIP; this mechanism does not operate in the mouse model because of the caspase block.

Embelin is a compound from the Japanese Ardisia herb (Herba ardisiae japonicae) used as a key ingredient in several traditional Chinese anti-cancer recipes as well as a contraceptive (54–57). XIAP knock-out mice have been generated previously (73); however, XIAP deficiency had no effect on caspase activation in vitro.

Caspase-3 amplification pathway (58), provides evidence for this pathway in pancreatitis.

Of interest, although embelin only prevents XIAP binding to and inhibition of caspase-9 (54), it induced activation of all three caspases measured, i.e., caspases-3, -9, and -8. Caspase-3 is a downstream target of caspase-9 (21). In turn, activation of caspase-8 with embelin could be through the caspase-3 → caspase-8 amplification pathway (58), providing evidence for this pathway in pancreatitis.

Our results indicate that XIAP is a key caspase regulator in pancreatitis. In particular, high XIAP levels maintained in mouse cerulein pancreatitis block caspase activation in this model, whereas XIAP degradation renders caspase activation in rat cerulein pancreatitis. Mechanism of XIAP degradation in pancreatitis is yet to be determined. XIAP is an ubiquitin ligase (E3 ligase) that can promote its own degradation (72); XIAP degradation can also be mediated by Smac/DIABLO, another ubiquitin ligase (25). XIAP knock-out mice have been generated previously (73); however, XIAP deficiency had no effect on caspase activation due to a compensatory up-regulation of other IAPs such as c-IAP1 and -2 (73).

Of note, caspase activation induced by embelin (e.g., 3-fold for caspase-9) was much less than that observed in rat cerulein pancreatitis. This suggests the involvement of other mechanisms, in addition to XIAP, in mediating the caspase block in mouse cerulein pancreatitis.

To determine the role of caspases in the regulation of cell-death responses in cerulein pancreatitis we used two approaches, namely, caspase inhibition in the rat model and caspase induction (with embelin) in the mouse model. Specific caspase inhibitors decreased apoptosis; conversely, caspase induction with embelin markedly increased apoptosis in the mouse model. These results indicate that caspases mediate apoptosis in cerulein pancreatitis. This conclusion is not obvious, because recent findings established that in many situations apoptosis develops without caspase activation (14, 16, 18, 74, 75).

We found that caspases not only mediate apoptosis but also protect from necrosis in cerulein pancreatitis. Caspase inhibition markedly stimulated necrosis in the rat model; conversely, caspase induction with embelin decreased necrosis in the mouse model. Similarly, our in vitro data (29) showed that caspase inhibition stimulated necrosis in isolated rat pancreatic acinar cells. These results provide an explanation for the observations of an inverse correlation between necrosis and apoptosis in experimental models of acute pancreatitis (Fig. 1 and Refs. 6–12).

Further, caspase inhibition in the rat model not only increased necrosis but also worsened other parameters of cerulein pancreatitis, i.e., serum levels of amylase and lipase, inflammatory infiltration in the pancreas, and histological changes. Conversely, embelin treatment improved pancreatic histology.

Mechanisms mediating necrosis are, in general, much less established than those for apoptosis. Recent findings indicate that there are two distinct types of necrosis (15–17). Accidental necrosis is triggered by severe cellular stress that is often characterized by depletion of ATP. By contrast, the so-called programmed necrosis, or necrosis-like programmed cell death, is mediated by specific signals. Our data show that necrosis in mouse cerulein pancreatitis develops rapidly: by 30 min after start of cerulein treatment, the extent of necrosis in this model increased from zero to ~5% (i.e. to the level observed in fully developed rat cerulein pancreatitis). At this time point, there was no ATP decrease (data not shown, and Refs. 76 and 77) and no inflammatory infiltration in the pancreas (5, 38, 78). These data provide evidence for the involvement of programmed necrosis in cerulein pancreatitis.

Our results further suggest the involvement of RIP, a mediator of programmed necrosis (15–17, 32–34), in the regulation of cell-death responses of pancreatitis. We found that the behavior of RIP drastically differed between the two models of cerulein pancreatitis. RIP underwent rapid and complete cleavage in the rat model but remained intact in the mouse model. RIP degradation in rat cerulein pancreatitis was inhibited by caspase inhibitors, whereas caspase induction with embelin
triggered RIP cleavage in the mouse model. These data indicate that RIP is regulated by caspases in cerulein pancreatitis.

The level of intact RIP directly correlated with the extent of necrosis in cerulein pancreatitis. Maximal RIP cleavage was observed in rat cerulein pancreatitis that has little necrosis, whereas there was no RIP degradation in the mouse cerulein and rat arginine pancreatitis, the models characterized by high necrosis.

The results of the present study show that acinar cell death in pancreatitis is regulated by a number of signaling mechanisms the balance of which determines the pattern of parenchymal cell death, i.e. apoptosis versus necrosis. These mechanisms, and their different involvement in the rat versus mouse models of cerulein pancreatitis, are depicted in Fig. 11. Caspase activation is a major factor in this balance, switching the cell-death response toward apoptosis and away from necrosis. Our results indicate XIAP as a key negative regulator of caspases in pancreatitis. Maintaining intact XIAP levels (and, possibly, levels of other IAPs) results in a reduction in the level of cleaved XIAP, which is accompanied by reduced caspase activation and reduced cell death.

The findings in the present study raise a number of further questions about the mechanisms of death responses in pancreatitis. In particular, do the signaling mechanisms that we found to regulate cell-death responses of cerulein pancreatitis (i.e. caspases, XIAP, and RIP) also operate in other experimental models of acute pancreatitis, such as the CDE (choline-deficient, ethionine-supplemented) diet or duct ligation models (37), as well as in human disease? What pathways mediate caspase-independent apoptosis in pancreatitis? What is the mechanism of XIAP degradation? Is there a role for other IAPs in the regulation of cell death in pancreatitis? What are the downstream targets of RIP in pancreatitis?

In sum, our results demonstrate key roles for caspases, XIAP, and RIP in the regulation of cell-death responses of pancreatitis. They show how manipulating death-signaling mechanisms changes the necrosis/apoptosis pattern in experimental pancreatitis. These signals represent potential therapeutic targets in the treatment of pancreatitis, especially to prevent or attenuate necroinflammatory processes.

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