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ORIGINAL RESEARCH

Necroptosis Is an Important Severity Determinant and Potential Therapeutic Target in Experimental Severe Pancreatitis

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SUMMARY

During experimental acute pancreatitis, pancreatic acinar cells die primarily because of necroptosis. Inhibition of necroptosis either by administration of necrostatin or by genetic manipulation ameliorates the severity of acute pancreatitis. Delayed pharmacologic inhibition of necroptosis also reduces disease severity.

BACKGROUND & AIMS: Severe acute pancreatitis is characterized by acinar cell death and inflammation. Necroptosis is an aggressive and proinflammatory mode of cell death that can be prevented by necrostatin-1 administration or receptor-interacting protein kinase (RIP3) deletion.

METHODS: Mouse pancreatic acinar cells were incubated with supramaximally stimulating concentrations of caerulein or submicellar concentrations of taurolithocholic acid-3-sulfate (TLCS), and necroptosis was inhibited by either addition of necrostatin or by RIP3 deletion. Cell death was quantitated using either lactate dehydrogenase leakage from acini or propidium iodide staining of nuclei. Necrosome formation was tracked and quantitated using cell fractionation or immunoprecipitation. Pancreatitis was induced in mice by retrograde intraductal infusion of TLCS or by repetitive supramaximal stimulation with caerulein.

RESULTS: Necroptosis was found to be the most prevalent mode of acinar cell in vitro death and little or no apoptosis was observed. Acinar cell death was associated with necrosome formation and prevented by either necrostatin administration or RIP3 deletion. Both of these interventions reduced the severity of TLCS- or caerulein-induced pancreatitis. Delaying necrostatin administration until after pancreatitis already had been established did not prevent its ability to reduce the severity of TLCS-induced pancreatitis.

CONCLUSIONS: Necroptosis is the predominant mode of acinar cell death in severe experimental mouse pancreatitis. The severity of pancreatitis can be reduced by administration of necrostatin, and necrostatin still can reduce the cell injury of pancreatitis even if it is administered after the disease already has been established. Inhibition of necroptosis may be an effective strategy for the treatment of severe clinical pancreatitis. *(Cell Mol Gastroenterol Hepatol 2016;2:519–535; http://dx.doi.org/10.1016/j.jcmgh.2016.04.002)*

Keywords: Acute Pancreatitis; Biliary Pancreatitis; Necroptosis; Apoptosis; Pancreatic Cell Death.

Acute pancreatitis is a relatively common but poorly understood inflammatory disease involving the exocrine pancreas. To date, no specific treatment for acute pancreatitis has been identified. The majority of patients with mild acute pancreatitis recover quickly without specific treatment, but roughly 20% of patients with severe pancreatitis, most of whom have evidence of pancreatic necrosis, experience significant morbidity and a mortality rate that can approach 20%.[1,2] The mechanisms responsible for pancreatic necrosis in acute pancreatitis and the basis for the high mortality rate in the subgroup of patients with severe necrotizing pancreatitis are poorly understood, but a better understanding of these phenomena and identification of interventions that could reduce the severity of pancreatitis are likely to have considerable impact on the treatment and outcome of this relatively common disease.

Until recently, only 2 forms of cell death had been recognized: a regulated programmed form of cell death, referred to as *apoptosis*, and an unregulated or accidental form of cell death, loosely referred to as *necrosis*. Apoptosis can be mediated by either extrinsic or intrinsic mechanisms, and most apoptotic cell death involves caspase-dependent events. It is characterized morphologically by cell shrinkage and other characteristic cell changes in the absence of an inflammatory response. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining and measurement of caspase activity are 2 of many methods of timing and quantitating the onset and extent of apoptosis. Apoptosis also is sensitive to inhibition by the pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-fmk) (reviewed by Galluzzi et al[3]).

Abbreviations used in this paper: **AMC, 4-amido-methylcoumarin; ATP, adenosine triphosphate; BAPTA, 2-bis (2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; IL, interleukin; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; MLKL, mixed-lineage kinase like; PI, propidium iodide; RIP, receptor-interacting protein kinase; TBS, Tris-buffered saline; TLCS, taurolithocholic acid-3-sulfate; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; Z-DEVD, benzyloxycarbonyl-Asp-Glu-Val-Asp-aminomethylcoumarin.**

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In contrast to apoptosis, necrosis is characterized morphologically by a gain in cell volume, swelling of organelles, plasma membrane rupture, extravasation of intracellular contents, and the presence of an acute inflammatory response.[4] In the past, necrosis was thought to always be an accidental and uncontrollable mode of cell death, but recent observations have indicated that some forms of cell death that morphologically appear to be necrotic are, in fact, finely regulated by a set of intracellular signal transduction pathways.[5] The best-characterized and most widely studied of these regulated forms of necrosis is necroptosis.[3] Necroptosis has been observed to occur after ligation of death domain receptors (eg, binding of tumor necrosis factor-α to tumor necrosis factor–receptor 1) and the process of necroptosis is known to involve the activation and translocation of the receptor-interacting protein kinase (RIP)1 and RIP3 kinases and the pseudokinase mixed-lineage kinase like (MLKL) to a large, amyloid-like, multimolecular scaffold complex dubbed the *necrosome*.[6–9] By incompletely understood mechanisms, the assembled necrosome then can mediate necroptotic cell death. In some cases, necroptosis is thought to be autophagy-dependent,[10] however, by definition, it is always dependent on activation of the kinase RIP1[3,11] and it can be inhibited by either pharmacologic inhibition of RIP1 (eg, by small molecules known as necrostatins[12,13]) or by genetic deletion of RIP3.[1] Genetic deletion of RIP1 is embryonically lethal, but it now is known that activation of RIP1 depends on its association with RIP3 kinase[3,11] and, fortunately, genetic deletion of RIP3 is not embryonically lethal. As a consequence, prevention of RIP1 activation (followed by prevention of necrosome formation and necroptosis) can be accomplished experimentally by genetic deletion of RIP3. Recent data have shown that MLKL functions as the executioner molecule in necroptosis, targeting phosphatidylinositol binding sites and rupturing the plasma membrane.[14] The consequences of preventing necroptotic cell death appear to be cell- and disease state–specific. In some cases, it appears to promote alternative forms of cell death such as apoptosis, however, in other cases, prevention of necroptosis leads to events that favor cell survival.[13]

In the current article, we report the results of studies designed to examine the mode of pancreatic acinar cell death during the early stages of 2 experimental mouse models of severe pancreatitis: bile acid–induced and secretagogue-induced severe pancreatitis. Our studies had the following 4 primary goals: (1) to identify the most prevalent mode of cell death during the early stages of these 2 models of severe pancreatitis; (2) to examine the sequence and timing of some of the events related to acinar cell necroptotic cell death during the evolution of severe pancreatitis; (3) to define the effects on pancreatitis severity of inhibiting this most prevalent mode of cell death; and (4) to examine the possibility that inhibiting this mode of cell death can reduce the severity of pancreatitis even if that inhibition of cell death occurs after the start of pancreatitis induction.

Materials and Methods

Materials

The amylase substrate (2-chloro-*p*-nitrophenyl-α-maltotrioside), the lactate dehydrogenase substrate (lactate), and the indicator for lactate dehydrogenase assay (nicotinamide adenine dinucleotide) were purchased from Sekisui Diagnostics Chemical Ltd (Exton, PA). Two-bis (2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), the caspase-3, -7 substrate Z-DEVD-AMC (benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin), and the pancaspase substrate rhodamine 110 bis (l-aspartic acid amide) (D2R110) were obtained from Life Technologies (Carlsbad, CA). The pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; (Z-VAD-fmk or ZVAD) and polyvinyldifluoride membranes were purchased from EMD Millipore (Billerica, MA). Ridaifen B, a tamoxifen analog that is a known inducer of apoptosis,[15] was purchased from Sigma-Aldrich (St. Louis, MO). The luciferase-based adenosine triphosphate (ATP) assay was obtained from Perkin Elmer. Antibodies against RIP3 were purchased from Cell Signaling (Beverly, MA) while antibodies against total MLKL and phosphorylated MLKL were from Abcam (Cambridge, MA). Antibodies against RIP1 were from ProSci (Poway, CA). Dulbecco's modified Eagle medium mixed 1:1 with Ham's F12 salts (DMEM-F12) and sodium pyruvate were from Life Technologies. Nitex mesh filters were purchased from Sefar America (Kansas City, MO). Necrostatin-1 was obtained from Enzo Life Sciences (Farmingdale, NY). Caerulein was purchased from Bachem (Torrance, CA). The TUNEL-staining kit was purchased from Millipore (Waltham, MA). For immunoprecipitation we used the Classic Magnetic IP/Co-IP kit from ThermoFisher Scientific (Waltham, MA). The enzyme-linked immunosorbent assays for monocyte chemoattractant protein 1 (MCP-1) and interleukin (IL)6 were purchased from R&D Systems, Minneapolis, MN). Taurolithocholic acid 3-sulfate disodium salt (TLCS), propidium iodide and all other chemicals were of analytic grade and purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Animals

All experiments were performed using non–sex-selected, wild-type C57Bl/6 mice (20–30 g) purchased from Jackson Labs (Bar Harbor, ME), or 20–30 g *RIP3*$^{-/-}$ mice, of either sex, that had been bred from founder C57Bl/6 knock-out animals kindly donated by Dr Xiaodong Wang (University of Texas Southwestern Medical Center).[16] These mice were back-crossed to a C57Bl/6 background for 10 generations. The animals were housed in temperature-controlled (23°C ± 2°C) rooms with a 12:12-hour light:dark cycle, fed standard laboratory chow, and allowed water ad libitum. All experiments were performed according to protocols approved by the Animal Care and Use Committee of the Tufts Medical Center.

Preparation of Pancreatic Acini and Fragments

Pancreatic acini (1–100 cells per cluster) were freshly prepared for each experiment using collagenase digestion in

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