Pathophysiology of Alcohol-Induced Pancreatitis

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Abstract: Excessive ethanol consumption is a common risk factor for acute and chronic pancreatitis. Ethanol could lead to the onset of pancreatitis in a number of ways; the most recently discovered is its effect on intrapancreatic digestive enzyme activation, by either sensitizing acinar cells to pathologic stimuli or stimulating the release of a secretagogue (cholecystokinin) from duodenal I cells. Recent advances in cell biologic and molecular techniques have permitted us to address the intracellular events involved in digestive enzyme activation in a manner that was previously considered impossible. Investigations that used these novel techniques found that (a) trypsin is, in contrast to its role in the small intestine, not necessarily involved in the premature intracellular activation of other digestive proteases such as proelastase; (b) trypsinogen does not autoactivate intracellularly but is instead largely activated by the lysosomal hydrolase cathepsin B; and (c) the role of trypsin in the intrapancreatic protease cascade is most likely one that involves the degradation, rather than the activation, of active digestive proteases including trypsin itself. These studies, as well as investigations that have addressed the role of mutant trypsin in the disease onset of hereditary pancreatitis, suggest that trypsin may not be critical for triggering pancreatitis but might have a protective role against the action of some of the other digestive proteases. While the specific role of different digestive enzymes in initiating pancreatitis is still a matter of debate and the topic of ongoing investigations, experimental evidence suggests that ethanol can directly interfere with the processes involved in digestive zymogen activation.

Key Words: trypsinogen, trypsinogen activation peptide, cholecystokinin, ethanol, serine proteases

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The pancreas synthesizes and secretes its digestive proteases mostly as inactive zymogens that need to be activated in the small intestine to catalyze the breakdown of food proteins. More than 100 years ago, it was proposed that pancreatitis is essentially a disease in which the pancreas falls prey to its own, prematurely activated digestive enzymes. Where and why digestive zymogens become prematurely and intracellularly activated within the pancreas during the initiating phase of acute pancreatitis has been the topic of extensive research efforts and debate. This article reviews the most recent developments in this field and will specifically focus on the role of trypsin and cathepsin B in the intracellular activation cascade that precedes acinar cell injury. We also review the studies in which a possible involvement of ethanol in these processes has been documented.

The exocrine pancreas synthesizes and secretes more protein per cells than any other exocrine organ. Much of its protein secretion consists of digestive proenzymes, called zymogens, that require cleavage of an activation peptide by a protease. After entering the small intestine, the pancreatic zymogen trypsinogen is first processed to trypsin by the intestinal protease enterokinase. Trypsin then proteolytically processes the other pancreatic enzymes to their active forms. Therefore, under physiological conditions, pancreatic proteases remain inactive during their synthesis, intracellular transport, secretion from acinar cells, and transit through the pancreatic duct. They only become active when reaching the lumen of the small intestine. About a century ago, the pathologist Hans Chiari suggested that the pancreas of patients who had died during episodes of acute necrotizing pancreatitis "had succumbed to its own digestive properties," and he postulated that pancreatic "autodigestion" is the underlying pathophysiological mechanism of the disease.[1] Since then, many attempts have been made to prove or disprove the role of premature, intracellular zymogen activation as an initial or an initiating event in the course of acute pancreatitis. Only recent advances in biochemical and molecular techniques have allowed investigators to address some of these questions conclusively.

Much of our current knowledge regarding the onset of pancreatitis was not gained from studies involving the human pancreas or patients with pancreatitis but came from animal and isolated cell models. There are several reasons why these

models have been used. (a) The pancreas is a rather inaccessible organ because of its anatomic location in the retroperitoneal space. Biopsies of human pancreas are difficult to obtain for ethical and medical reasons, unlike colon or stomach biopsies. (b) Patients who are admitted to the hospital with acute pancreatitis have usually passed through the initial stages of the disease when the triggering early events could have been studied. The autodigestive process that characterizes this disease has remained a particularly significant impediment for investigations that address initiating pathophysiological events.

The issue of premature protease activation has, therefore, mostly been studied in animal models of the disease.[2–4] These models can be experimentally controlled, are highly reproducible, and recapitulate many of the cellular events that are associated with clinical disease.

THE MECHANISM OF INTRAPANCREATIC ZYMOGEN ACTIVATION

Issues relating to the site and mechanisms responsible for the initiation of pancreatitis have not been easily resolved. Early hypotheses were based on autopsy studies of patients who had died in the course of pancreatitis. An early theory based on morphologic studies of human material suggested that peripancreatic fat necrosis represented the initial event from which all later alterations were derived.[5] This hypothesis was attractive because it implicated pancreatic lipase as the culprit for pancreatic necrosis. This enzyme is secreted from acinar cells in its active form and does not require activation by brush border hydrolases. Another hypothesis suggested that periductal cells represented the site of initial damage and that the extravasation of pancreatic juice from the ductal system was responsible for initiating the disease.[6] Subsequent controlled studies performed in animal models that simulate human disease have demonstrated that the acinar cell is the initial site of morphologic damage.[7] This conclusion has been supported by genetic studies that have linked the hereditary form of pancreatitis to mutations in the trypsinogen gene.[8,9]

Trypsinogen and other pancreatic proteases are synthesized in the acinar cell as inactive proenzyme precursors and stored in membrane-confined zymogen granules. After activation in the small intestine, trypsin converts other pancreatic zymogens such as proelastase, procarboxypeptidase, and prophospholipase A_2 to their active forms.[10] Though small amounts of trypsinogen are probably activated within the pancreatic acinar cell under physiological conditions, several protective mechanisms normally prevent cell damage from proteolytic activity. The protective mechanisms within the zymogen granules include (a) the presence of large amounts of pancreatic trypsin inhibitor, (b) an acidic pH within the distal secretory pathway, including the zymogen granule, that is below the optimum for most proteolytic enzymes, and (c) the presence of proteases that can degrade other already active proteases. However, premature activation of large amounts of trypsinogen could potentially overwhelm these protective mechanisms. This may lead to damage of the zymogen-confining membrane and release of activated zymogens into the cytosol. Moreover, release of large amounts of calcium from the zymogen granule into the cytosol may activate calcium-dependent proteases such as calpains that contribute to cell injury.

The suggestion that zymogen activation plays a central role in the pathogenesis of pancreatitis is based on the following observations: (a) the activity of both pancreatic trypsin and elastase increases early in the course of experimental pancreatitis,[11,12] (b) the activation peptides of trypsinogen and carboxypeptidase A_1 (CPA_1), which are cleaved from the respective proenzyme during the process of activation, is released into the serum early in the course of acute pancreatitis,[13] (c) pretreatment with gabexate mesylate, a serine protease inhibitor, reduces the incidence of ERCP-induced pancreatitis,[14,15] (d) serine protease inhibitors reduce injury in experimental pancreatitis,[16,17] and (e) forms of the hereditary variety of pancreatitis are associated with mutations in the trypsinogen gene that could render trypsinogen more prone to premature activation or may render active trypsin more resistant to degradation by other proteases.[18,19] These observations provide compelling evidence that premature, intracellular zymogen activation plays a critical role in initiating acute pancreatitis.

Clinical and experimental studies demonstrate that zymogen activation is a very early feature of the disease. The activation peptides of trypsinogen and carboxypeptidase A_1, both markers for zymogen activation, can be detected in the serum within the first hours of acute pancreatitis.[20,21] Similarly, trypsin activity and immunoreactivity for the trypsinogen activation peptide (TAP) increase rapidly (within 15 minutes) after the induction of experimental pancreatitis.[12,22] One study that used the cerulein model of acute pancreatitis reported a biphasic pattern of trypsin activity that reached an early peak after 1 hour and a second peak several hours later.[12] This observation suggests that more than one mechanism may be involved in the activation of pancreatic zymogens. The clinical importance or the mechanism of the second peak of trypsin activity is unknown, but it may require inflammation.[23]

EVIDENCE OF ZYMOGEN ACTIVATION FROM CLINICAL AND GENETIC STUDIES

Several recent studies involving patients have greatly contributed to understanding the role of zymogen activation in pancreatitis. A clinical trial in which a small molecular weight protease inhibitor was administered to patients before they underwent ERCP reduced the incidence of ERCP-related pancreatitis.[14] Although protease inhibitors have been shown to be ineffective when used therapeutically in patients with clinically established pancreatitis,[24] the result of the ERCP study suggests that the activation of pancreatic proteases is an inher-

ent feature of the onset of the disease and that its prevention could potentially lower the incidence of pancreatitis. Moreover, since reasonably specific antibodies have become available that detect TAP but do not cross-react with either active trypsin or inactive trypsinogen,[25] the presence of TAP in the serum and urine of patients with acute pancreatitis provides direct proof of the activation of trypsinogen during pancreatitis, and the amount of TAP released also appears to correlate with the disease severity.[26]

A very different line of evidence comes from studies of molecular alterations associated with the hereditary form of pancreatitis. In families affected by this autosomal dominant disorder, the disease phenotype has been found to segregate with point mutations in the cationic trypsinogen gene.[8,27] Regarding the role of premature zymogen activation, the data from hereditary pancreatitis currently available are not always conclusive. Because trypsin activation is an event known to occur in pancreatitis[21] and because trypsin can activate many other digestive proteases of the pancreas in vitro,[10] previous attempts to interpret the functional consequences of trypsinogen mutations have focused on features that would either allow premature intracellular activation of trypsinogen or permit extended intracellular activity of trypsin. In a later section, we focus on the functional consequences of cationic trypsinogen mutations for the intracellular proteolytic cascade in the pancreas.

THE ROLE OF CATHEPSIN B IN PREMATURE ZYMOGEN ACTIVATION

Several earlier studies had suggested a role for cathepsin B in zymogen activation.[28,29] The observations supporting a cathepsin hypothesis include the following: (a) cathepsin B can activate trypsinogen in vitro,[30,31] (b) cathepsin B is redistributed to a zymogen granule–enriched subcellular compartment,[32] and (c) lysosomal enzymes colocalize with digestive zymogens during the early course of experimental pancreatitis.[33] Although this cathepsin hypothesis appears attractive from a cell biologic point of view and valid alternative hypotheses are lacking, it has received much criticism, and the following experimental observations appear to be incompatible with its assumptions: (a) a colocalization of cathepsins with digestive zymogens has not only been observed in the initial phase of acute pancreatitis but also under physiological control conditions and in secretory vesicles that are destined for regulated secretion from healthy pancreatic acinar cells,[34,35] (b) a redistribution of cathepsin B into a zymogen-enriched subcellular compartment can be induced in vivo by experimental conditions that interfere with lysosomal sorting and are neither associated with nor followed by the development of acute pancreatitis,[36] (c) the administration of potent lysosomal enzyme inhibitors in vivo does not prevent the onset of acute experimental pancreatitis,[37] (d) both increases and decreases in the rate of intracellular trypsinogen activation have been reported in experiments that used lysosomal enzyme inhibitors in vitro,[38,39] and even a protective role against a premature zymogen activation has been considered for cathepsin B.[40,41]

In view of the limited specificity and bioavailability of the existing inhibitors for lysosomal hydrolases, the only remaining option for addressing the cathepsin hypothesis conclusively was, therefore, to generate cathepsin B–deficient animals. When we studied experimental pancreatitis in a strain of mice in which the cathepsin B gene had been deleted by targeted disruption, we found that the course of acute pancreatitis was altered in a number of ways.[42] The most dramatic change in comparison with wild-type control animals and the most relevant in regard to the cathepsin hypothesis of acute pancreatitis was a reduction in premature, intrapancreatic trypsinogen activation. In terms of substrate-defined trypsin activity, this reduction amounted to >80% over the course of 24 hours, and when the greater pancreatic trypsinogen content of cathepsin B knockout animals was taken into account, <10% of the relative amount of trypsinogen detected in wild-type animals was activated during the course of pancreatitis in cathepsin B knockout mice. This observation alone can be regarded as the first direct experimental evidence of a critical role of cathepsin B in the intracellular events that determine premature zymogen activation and the onset of acute pancreatitis. Surprisingly, the decrease in trypsinogen activation was not paralleled by a complete prevention of pancreatitis and pancreatic apoptosis, and the systemic inflammatory response of pancreatitis in the pancreas and lungs of the animals was not affected at all. This observation and the fact that cathepsin B is known to activate pancreatic digestive zymogens other than trypsinogen[43] raise the possibility that cathepsin B–induced trypsinogen activation may not be the sole cause of or may not be directly involved in acinar cell injury. Although most recent studies confirm that cathepsin B is abundant in the human pancreatic duct and the secretory compartment of human pancreatic acinar cells, its capacity to activate trypsinogen is not affected by the common trypsinogen mutations observed in association with hereditary pancreatitis.[44]

THE ROLE OF TRYPSIN IN PREMATURE ZYMOGEN ACTIVATION

Why structural changes in the cationic trypsinogen gene lead to the onset of hereditary pancreatitis has been a matter of debate. Since pancreatitis has long been regarded as a disease that is caused by proteolytic autodigestion of the organ[1] and because trypsin is known to be a potent activator of pancreatic zymogens in the gut,[10] it has been suggested that the trypsinogen mutations that were found in association with hereditary pancreatitis confer a gain of enzymatic function.[8,9] In vitro studies have analyzed the biochemistry of recombinant human trypsinogens into which pancreatitis-associated mutations were introduced and found that either facilitated trypsinogen autoactivation or extended trypsin activity can result under de-

fined experimental conditions.[45–48] Whether these experimental conditions reflect the highly compartmentalized situation in which protease activation begins intracellularly in vivo[49,50] is currently unknown, but the above studies would strongly suggest that either more effective autoactivation of trypsinogen or impaired inactivation of trypsin (by degradation or autolysis) would be involved in the onset of hereditary pancreatitis. Several arguments, however, have been raised against the gain of trypsin function hypothesis of hereditary pancreatitis. Statistically, most hereditary disorders are associated with loss-of-function mutations that render a specific protein either defective or impair its intracellular processing and targeting.[51] Moreover, at least 5 mutations, A16V,[52] D22G,[45] K23R,[53] N29I,[54] and R122H,[8] have been found in association with hereditary pancreatitis. These are located in different regions of the PRSS1 gene and would thus be expected to have different structural effects on the trypsinogen molecule. It would, therefore, be easier to explain their common pathophysiology in terms of a loss of enzymatic function rather than a gain of enzymatic function, especially since one of these mutations (A16V) also affects the signal peptide cleavage site that is assumed to be involved in the correct processing of trypsinogen.[52] Experiments in isolated pancreatic acini and lobules that studied the in vivo mechanisms of intracellular zymogen activation have shown that trypsin activity is neither required nor involved in the activation of other digestive proteases and that its most prominent role is in autodegradation.[55] This, in turn, would suggest that intracellular trypsin activity has a role in the defense against other potentially more harmful digestive proteases and that structural alterations that impair the function of trypsin would eliminate a protective mechanism rather than generate a triggering event for pancreatitis.[56] Whether these experimental observations obtained from rodent pancreatic acini and lobules have any relevance to human hereditary pancreatitis is currently unknown because human cationic trypsinogen has distinct characteristics in terms of its ability to autoactivate and to autodegrade.

A recently reported kindred with hereditary pancreatitis that carries a R122C mutation is very interesting in this context.[57] The single nucleotide exchange in this family is only 1 position upstream from the one found in the most common variety of hereditary pancreatitis and leads to an amino acid exchange at the same codon (R122C versus R122H).

When equal amounts of recombinant protein are used for biochemical studies, the enterokinase-induced activation and the autoactivation of Cys-122 trypsinogen are found to be significantly reduced by 60%–70% compared with the wild-type enzyme. A possible interpretation of these results would be that Cys-122 trypsinogen misfolds or forms mismatched disulfide bridges under intracellular in vivo conditions, which confers a dramatic loss of trypsin function that cannot be compensated for by facilitated autoactivation. If this scenario reflects the in vivo conditions within the pancreas, it would represent the first direct evidence from a human study of a potential protective role of trypsin activity in pancreatitis.[57] The question of whether the gain-of-function hypothesis or the loss-of-function hypothesis correctly predicts the pathophysiology of hereditary pancreatitis cannot currently not be resolved, short of direct access to living human acini from carriers of PRSS1 mutations or a transgenic animal model into which the human PRSS1 mutations have been introduced. The data from studies on rodent pancreatic acini and lobules, however, suggest that the role of trypsin in the onset of acute or chronic pancreatitis may be rather different than previously assumed.

THE ROLE OF ETHANOL IN PREMATURE ZYMOGEN ACTIVATION

A continuous or excessive consumption of ethanol is one of the most common risk factors for the development of acute, and even more specifically, chronic pancreatitis in humans. The mechanisms that are involved in alcohol-induced pancreatitis in general have been extensively studied in ethanol-fed laboratory animals. Although several cell biologic changes have been reported from these studies, their relevance to the human disease is questionable because neither rats nor mice develop pancreatitis when put on a high-alcohol diet for extended periods of time. Theoretically, ethanol might promote its damaging effect on pancreatic acinar cells through 2 mechanisms. First, ethanol may directly sensitize the acinar cell to a pathologic stimulus,[58] and second, ethanol may stimulate the release of a physiological secretagogue, cholecystokinin (CCK), from duodenal I cells.[59]

Recent studies in which the effect of ethanol on the intracellular activation of digestive protease zymogen was studied were rather successful in eliciting potentially disease-relevant mechanisms. Several studies found that ethanol can, indeed, sensitize acinar cells to CCK-induced procarboxypeptidase A_1 processing in vitro and can sensitize to various forms of pancreatitis in vivo.[60–63] Thus, ethanol is believed to enhance the stimulation-dependent induction of pancreatitis, and this process may play a role in ethanol toxicity in the pancreas. A recent study addressed several issues relevant to zymogen activation and the effects of ethanol in isolated pancreatic acini.[64] These authors observed that trypsinogen and chymotrypsinogen exhibit distinct patterns of activation in response to supraphysiological concentrations of the CCK analogue cerulein. Moreover, they found that ethanol and other alcohols sensitized the acinar cell to cerulein-induced trypsin and chymotrypsin activation and that other short-chain n-aliphatic alcohols (methanol, propanol, and butanol) enhanced the effects of cerulein on the acinar cell. Ethanol alone, on the other hand, was not found to induce pancreatitis or zymogen activation in experimental models of pancreatitis. These studies suggest that CCK receptor activation can initiate different patterns of zymogen activation in pancreatic acinar cells and the extent of activation can be enhanced by a distinct set of short-chain al-

cohols. Whether ethanol and other alcohols mediate these effects by interfering with acinar cell signaling pathways or by effecting acinar cell membrane fluidity is currently being investigated.

CONCLUSION

Recent advances in cell biologic and molecular genetic techniques have permitted the intracellular pathophysiology of pancreatitis to be addressed in a much more direct manner than was previously considered possible. Initial studies that have employed these techniques have delivered a number of surprising results that appear to be incompatible with long-standing dogmas and paradigms of pancreatology. Some of these insights will lead to new and testable hypotheses that will bring us closer to understanding the pathogenesis of pancreatitis. Only progress in elucidating the intracellular and molecular mechanisms involved in the disease onset will permit the development of effective strategies to prevent and cure this debilitating disease. While the specific role of trypsin and other digestive proteases in initiating pancreatitis is currently being investigated and still a matter of debate, it has already been established that ethanol can directly interfere with the processes that are involved in digestive zymogen activation. Future studies will show whether this is the critical link between ethanol toxicity and the onset of pancreatitis.

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