27 Trypsin Activation and Inhibition in Pancreatitis

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Pancreatitis is a common disorder in which the passage of gallstones and the consumption of immoderate amounts of alcohol induce an inflammatory process. Recent studies involving animal and isolated cell models have elucidated many of the pathophysiological, cellular and molecular processes involved in the disease onset. More than a century ago it was proposed that pancreatic digestive enzymes are involved in the onset of pancreatitis and that the disease is essentially the result of an auto-digestion of the gland. Why and how digestive zymogens undergo activation, in spite of numerous protective mechanism, has been the topic of intense research efforts and debate. In this chapter we review the most recent progress in this enterprise and will specifically focus on mechanisms involved in gallstone-induced and hereditary pancreatitis.

Etiology and Pathogenesis of Pancreatitis

Pancreatitis is a common disease with an incidence of approximately 25/100 000 population per year. It differs from other inflammatory disorders in that infectious agents and autoimmune processes are considered exceedingly rare causes of the disease. The mild form of acute pancreatitis, which accounts for some 75% to 80% of cases has virtually no mortality and patients recover more or less spontaneously whereas the severe form is characterized by local and systemic complications, may lead to multi-organ failure and is burdened with a mortality rate of between 5 and 20 %. No specific treatment for pancreatitis is known today. The most common etiological factors are alcohol abuse and gallstone migration which, together, account for more than 80% of cases with acute pancreatitis in most Western countries. While the mechanisms involved in causing pancreatitis through alcohol consumption are still being explored and poorly understood much progress has been made in elucidating the role of gallstones in the pathophysiology of acute pancreatitis. We will therefore begin our review with some of the recent advances in understanding gallstone-induced pancreatitis.

Pathophysiology of Gallstone-induced Pancreatitis

Claude Bernard discovered in 1856[1] that bile is an agent that, when injected into the pancreatic duct of laboratory animals, can cause pancreatitis. Since that time

many studies have been performed to elucidate the underlying mechanisms. While it is firmly established today that the initiation of pancreatitis requires the passage of a gallstone from the gallbladder through the biliary tract,[2] whereas gallstones that remain in the gallbladder will not cause pancreatitis, the various hypotheses that were proposed to explain this association are often contradictory. In 1901 Eugene Opie postulated that impairment of the pancreatic outflow due to obstruction of the pancreatic duct causes pancreatitis.[3] This initial 'duct obstruction hypothesis' was somewhat forgotten when Opie published his second 'common channel' hypothesis during the same year.[4] This later hypothesis predicts that an impacted gallstone at the papilla of Vater creates a communication between the pancreatic and the bile duct (the said 'common channel') through which bile flows into the pancreatic duct and thus causes pancreatitis.

Although Opie´s 'common channel' hypothesis seems rational from a mechanistic point of view and has become one of the most popular theories in the field, considerable experimental and clinical evidence is incompatible with its assumptions.[5,6] Anatomical studies have shown that the communication between the pancreatic duct and the common bile duct is much too short (<6 mm) to permit biliary reflux into the pancreatic duct,[7] and an impacted gallstone would most likely obstruct both the common bile duct and the pancreatic duct.[8] Even in the event of an existing anatomical communication pancreatic secretory pressure would still exceed biliary pressure and pancreatic juice would flow into the bile duct rather than bile into the pancreatic duct.[9,10] Late in the course of pancreatitis when necrosis is firmly established a biliopancreatic reflux due to a loss of barrier function in the damaged pancreatic duct may well explain the observation of a bile-stained necrotic pancreas at the time of surgery. This, however, should not be regarded as evidence for the assumption that reflux of bile into the pancreas is an initial triggering event for the disease onset. Experiments performed on the American opossum, an animal model that is anatomically well suited to test the common channel hypothesis, have revealed that neither a common channel, nor biliopancreatic reflux is required for the development of acute necrotizing pancreatitis.[6]

In order to overcome the inconsistencies of the 'common channel' hypothesis it was proposed that the passage of a gallstone could damage the duodenal sphincter in a manner that results in sphincter insufficiency. This, in turn, could permit duodenal content, including bile and activated pancreatic juice, to flow through the incompetent sphincter and into the pancreatic duct[11] thus inducing pancreatitis. While this hypothesis would, indeed, avoid most of the inconsistencies of Opie's 'common channel' hypothesis it was shown not to be applicable to the human situation in which sphincter stenosis, rather than sphincter insufficiency, results from the passage of a gallstone through the papilla and flow of pancreatic juice into the bile duct, rather than flow of duodenal content into the pancreas, is the consequence.[12] A final argument against the 'common channel' hypothesis is that the perfusion of bile through the pancreatic duct has been shown to be completely harmless[13] and only a potential influx of infected bile, which might occur after prolonged obstruction at the papilla when the pressure gradient between the pancreatic duct (higher) and the bile duct (lower) is reversed,[14,15] may represent an aggravating factor, as opposed to an initiating event, for the course of pancreatitis.

Taken together these data suggest that the initial pathophysiological events during the course of gallstone-induced pancreatitis affect acinar cells[16] and are triggered, in accordance with Opie's initial hypothesis, by obstruction or

impairment of flow form the pancreatic duct.[17] A reflux of bile into the pancreatic duct – either through a common channel created by an impacted gallstone or through an incompetent spincter caused by the passage of a gallstone – is neither required nor likely to occur during the initial course of acute pancreatitis.

Cellular Events During Pancreatic Duct Obstruction

An animal model based on pancreatic duct obstruction in rodents was recently employed to investigate the cellular events involved in gallstone-induced pancreatitis.[18] In addition to a morphological and biochemical characterization of this experimental disease variety the intracellular calcium release in response to hormonal stimuli was investigated. Under physiological resting conditions most cell types, including the acinar cells of the exocrine pancreas, maintain a Ca^{2+}-gradient across the plasma membrane with low intracellular (nanomolar range) facing high extracellular (millimolar range) Ca^{2+}-concentrations. A rapid Ca^{2+}-release from intracellular stores in response to external and internal stimuli is used by many of these cells as a signaling mechanism that regulates such diverse biological events as growth and proliferation, locomotion and contraction, and the regulated secretion of exportable proteins. An impaired cellular capacity to maintain the Ca^{2+}-gradient across the plasma membrane has previously been identified as a common pathophysiological characteristic of vascular hypertension,[19,20] malignant tumor growth,[21,22] and cell damage in response to toxins.[23] It has also been observed in a secretagogue-induced model of acute pancreatitis.[24,25] Up to 6 h of pancreatic duct ligation in rats and mice, a condition that mimics the situation in human gallstone-induced pancreatitis, induced leukocytosis, hyperamylasemia, pancreatic edema and increased lung myeloperoxidase activity, all of which were not observed in bile duct-ligated controls.[18] It also led to a profound intracellular activation of pancreatic proteases such as trypsin, an event we will discuss in the next paragraph in more detail. In acinar cells of isolatd acini from these animals the resting $[Ca^{2+}]_i$ in isolated acini rose by 45% to 205±7 nmol, whereas the acetycholine- and cholecystokinin-stimulated calcium peaks as well as amylase secretion declined. However, neither the $[Ca^{2+}]_i$ signaling pattern nor the amylase output in response to the Ca^{2+}-ATPase inhibitor thapsigargin, nor the secretin-stimulated amylase release, were impaired by pancreatic duct ligation. On the single cell level pancreatic duct ligation reduced the percentage of cells in which a physiological secretagogue stimulation was followed by a physiological response (i.e. Ca^{2+}-oscillations) and increased the percentage of cells with a pathological response (i.e. peak-plateau or absent Ca^{2+}-signal). Moreover, it reduced the frequency and amplitude of Ca^{2+}-oscillation as well as the capacitative Ca^{2+}-influx in response to secretagogue stimulation.

To test whether these prominent changes in intra-acinar cell calcium signaling not only parallel pancreatic duct obstruction but are directly involved in the initiation of pancreatitis, animals were systemically treated with the intracellular calcium chelator BAPTA-AM. As a consequence, both the parameters of pancreatitis as well as the intrapancreatic trypsinogen activation induced by duct ligation were found to be significantly reduced. These experiments suggest that pancreatic duct obstruction, the critical event involved in gallstone-induced pancreatitis, rapidly changes the physiological response of the exocrine pancreas to a pathological Ca^{2+}-signaling pattern. This pathological Ca^{2+}-signaling is associated with

premature digestive enzyme activation and the onset of pancreatitis – both of which can be prevented by administration of an intracellular calcium chelator. We set out below why we and others believe that alterations in calcium signaling and the premature activation of digestive proteases represent such critical events in the onset of pancreatitis.

Mechanisms of Pancreatic Autodigestion

The exocrine pancreas synthesizes and secretes more protein per cell than any other exocrine organ. Much of its protein secretion consists of digestive proenzymes, called zymogens that require proteolytic 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 other pancreatic enzymes to their active forms. Under physiological conditions pancreatic proteases thus remain inactive during synthesis, intracellular transport, secretion from acinar cells, and transit through the pancreatic duct. They are activated only when reaching the lumen and brushborder 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 created the term "auto-digestion" to describe the underlying pathophysiological disease mechanism.[26] 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.

Experimental Models for Intrapancreatic Protease Activation

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 are being used: 1) The pancreas is a rather inaccessible organ due to its anatomical location in the retroperitoneal space. Biopsies of the human pancreas are difficult to obtain for ethical and medical reasons. 2) Patients who are admitted to the hospital with acute pancreatitis have already passed through the initial stages of the disease when the initial triggering events could have been studied. 3) The autodigestive process that characterizes this disease is a particularly significant impediment for investigations that attempt to address initiating pathophysiological events using autopsy or surgical specimens. The issue of premature protease activation has therefore been studied mostly in animal models of the disease.[27–29] These models can be experimentally controlled, are highly reproducible, and recapitulate many of the cellular events that are associated with the clinical disease.

Initial Cellular Site of Onset in Pancreatitis

The question where pancreatitis begins and through what mechanisms the disease is initiated has not been easily resolved. Early hypotheses were based on autopsy

studies of patients who had died in the course of pancreatitis. One of these early theories based on human autopsy material suggested that peripancreatic fat necrosis represents the initial event from which all later alterations arise.[30] This hypothesis was attractive because it implicated pancreatic lipase as the culprit for pancreatic necrosis. Lipase is secreted from acinar cells in its active form and does not require activation by brushborder enterokinase. Another hypothesis suggested that periductular cells represented the site of initial damage and that the extravasation of pancreatic juice from the ductal system is responsible for initiating the disease onset.[31] Subsequent controlled studies performed in animal models that simulate the human disease have demonstrated that the acinar cell is the initial site of morphological damage.[16] The conclusion that pancreatitis begins in exocrine acinar cells, as opposed to some poorly defined extracellular space, is important because it represents a shift from earlier mechanistic and histopathological interpretations of the disease onset and it led to cell biological investigations of the underlying causes of pancreatitis. The concept of acinar cells as the primary site of the disease onset is also supported by recent clinical and genetic data[32,33] that are summarized below.

PathoPhysiological Importance of Digestive Protease Activation

Trypsinogen and other pancreatic proteases are synthesized by acinar cells as inactive proenzyme precursors and stored in membrane-confined zymogen granules. After activation in the small intestine, trypsin converts other pancreatic zymogens such as pro-elastase, pro-carboxypeptidase, or pro-phospholipase A2 to their active forms.[34] Although small amounts of trypsinogen are probably activated within the pancreatic acinar cell under physiological conditions, a number of protective mechanisms normally prevent cell damage from proteolytic activity. These protective intracellular mechanisms include: a) the presence of large amounts of pancreatic secretory trypsin inhibitor (PSTI), the product of the SPINK1 gene, b) an acidic pH within the distal secretory pathway, including the zymogen granule, which is below the optimum for most digestive proteases, and c) the presence of proteases that degrade other already active proteases. Theoretically a premature activation of large amounts of trypsinogen could overwhelm these protective mechanisms, lead to damage of the zymogen-confining membranes and the release of activated proteases into the cytosol. Moreover, the release of large amounts of calcium from zymogen granules into the cytosol might activate calcium-dependent proteases such as calpains which, in turn, could contribute to cell injury.

The suggestion that prematurely activated digestive enzymes play 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,[35,36] b) the activation peptides of trypsinogen and carboxypeptidase A_1 (CPA_1), which are cleaved from the respective proenzyme during the process of activation, are released into either the pancreatic tissue or the serum early in the course of acute pancreatitis,[34,37–41] c) pretreatment with gabexate mesilate, a serine protease inhibitor, reduces the incidence of ERCP-induced pancreatitis,[42,43] d) serine protease inhibitors reduce injury in experimental pancreatitis[44,45] and e) hereditary pancreatitis is often associated with various mutations in the cationic trypsinogen gene that could render trypsinogen either

more prone to premature activation or may render active trypsin more resistant to degradation by other proteases.[46,47]

In clinical and experimental studies that investigated the time course of pancreatitis it was found that zymogen activation occurs very early in the disease course. One study that employed the caerulein model of acute pancreatitis reported a biphasic pattern of trypsin activity that reached an early peak after 1 h and a later second peak after several hours.[41] This observation is interesting because it suggests that more than one mechanism may be involved in the activation of pancreatic zymogens and the second peak may require the infiltration of inflammatory cells into the pancreas.[41] Taken together these observations represent compelling evidence that premature, intracellular zymogen activation plays a critical role in initiating acute pancreatitis.

Subcellular Site of Initial Protease Activation

One piece of information that would be critical for understanding the pathophysiological mechanisms involved in premature intrapancreatic protease activation would be the subcellular site where it begins. This question was addressed by three different approaches. Using a fluorogenic, cell permeant substrate specific for trypsin, confocal microscopy could clearly localize trypsinogen activation to the secretory compartment in acinar cell within minutes after supramaximal secretagogue stimulation, an *in vitro* situation that mimics a model of experimental pancreatitis.[48] When subcellular fractions containing different classes of secretory vesicles were subjected to density gradient centrifugation it was found that trypsinogen activation does not begin in mature zymogen granules but in membrane confined vesicles of lesser density that most likely correspond to immature condensing secretory vacuoles.[48] Experiments, in which antibodies directed against the activation peptide of trypsin (TAP) were used for ultrastructural immunocytochemistry electron microscopy, confirmed that the initial site of TAP generation and thus trypsinogen activation during experimental pancreatitis is, indeed, the secretory pathway. Again, within minutes of pancreatitis induction TAP was found in membrane confined secretory vesicles that were much less condensed than mature zymogen granules.[49] Taken together these data not only confirm that digestive protease activation begins within pancreatic acinar cells, as opposed to the pancreatic ducts or the interstitial space, but that mature zymogen granules in which digestive proteases are highly condensed are not necessarily the primary site of this activation. The first trypsin activity in acinar cells following a pathological stimulus is clearly detectable in membrane confined secretory vesicles in which trypsinogen, as well as lysosomal enzymes, are both physiologically present.

Clinical Role of Digestive Protease Activation

Recent studies involving patients have greatly contributed to understanding the role of zymogen activation in pancreatitis. In patients who underwent endoscopic retrograde cholangio-pancreatography (ERCP), the prophylactic administration of a small molecular weight protease inhibitor reduced the incidence of pancreatitis.[42,43] While protease inhibitors have not been found to be effective when used

therapeutically in patients with clinically-established pancreatitis,[50] the result of the prophylactic study supports the conclusion that activation of pancreatic proteases is an inherent feature of the disease onset. Moreover, since reasonably specific antibodies have become available that detect the trypsinogen activation peptide (TAP) but do not cross react with either active trypsin or inactive trypsinogen,[51] the presence of TAP in serum and urine of patients with acute pancreatitis provides direct evidence for an activation of trypsinogen during pancreatitis. The amount of TAP released also appears to correlate with the disease severity.[52]

Evidence from Genetic Studies

A very different line of evidence comes from studies of the genetic changes in pancreatitis. Most patients in which pancreatitis was found in association with a genetic risk factor either carry point mutations in the cationic trypsinogen gene[32,53,54] or in the most abundant protease inhibitor PSTI (SPINK1).[55–57] The fact that the most common disease-associated mutations found in pancreatitis patients so far involve either a digestive zymogen or its intracellular inhibitor leads to two conclusions: a) pancreatitis in humans, just as in animal models, begins not only within the pancreas but the initiating events must affect exocrine acinar cells which synthesize and store the mutated proteins and, b) digestive protease activation is, after all, a critical event that not only parallels pancreatitis but is directly involved in its onset. Which role individual proteases have in the activation cascades that eventually lead to acinar cell damage and necrosis is, however, another matter. While trypsinogen clearly undergoes activation during pancreatitis and all reported protease mutations associated with pancreatitis (16 in all) exclusively affect trypsin, its ultimate role in the disease onset may be more complex than previously assumed.

Cathepsin B in Premature Digestive Protease Activation.

Several lines of evidence have suggested a possible role for the lysosomal cystein protease cathepsin B in the premature and intrapancreatic activation of digestive enzymes reviewed (in Refs. 58,59). Observations that would support such a role of cathepsin B include the following: a) cathepsin B can activate trypsinogen *in vitro*,[60,61] b) subcellular fractionation experiments using animal tissue from experimental pancreatitis models indicate that cathepsin B is redistributed from its lysosomal to a zymogen-granule enriched subcellular compartment,[62] and c) lysosomal enzymes such as cathepsin D have been reported to colocalize with digestive zymogens in membrane-confined organelles during the early course of experimental pancreatitis.[63] Although the cathepsin hypothesis appears attractive from a cell biological point of view and testable alternative hypotheses have not been proposed, it has received much criticism because 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,[64,65] b) a redistribution of cathepsin B into a zymogen-enriched subcellular compartment can be induced *in vivo* by experimental conditions that inter-

fere with lysosomal sorting and are neither associated with, nor followed by, the development of acute pancreatitis,[66] c) the administration of potent lysosomal enzyme inhibitors *in vivo* does not prevent the onset of acute experimental pancreatitis,[67] d) both increases and decreases in the rate of intracelluar trypsinogen activation have been reported in experiments that used lysosomal enzyme inhibitors *in vitro*,[68,69] and e) even a protective role against a premature zymogen activation has been considered for cathepsin B.[70,71]

In view of the limited specificity and bioavailability of the available inhibitors for lysosomal hydrolases, the only remaining option to address the cathepsin hypothesis conclusively was therefore to generate cathepsin B deficient animals. When experimental pancreatitis in a strain of mice in which the cathepsin B gene had been deleted by targeted disruption was studied, the disease course was altered in a number of ways.[72] The most dramatic change in comparison to wild-type control animals, and also 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 more than 80% over the course of 24 h. When the greater pancreatic trypsinogen content of cathepsin B knock-out animals was taken into account, less than 10% of the amount of trypsinogen detected in wild-type animals was activated during the course of pancreatitis in the cathepsin B-deficient animals. This observation alone can be regarded as the first direct experimental evidence for a critical role of cathepsin B in the intracellular events that determine premature digestive protease activation during the onset of acute pancreatitis.

Surprisingly, the decrease in trypsinogen activation was not paralleled by a dramatic prevention of pancreatic necrosis, and the systemic inflammatory response during pancreatitis was not affected at all. This observation and the fact that cathepsin B can activate pancreatic digestive zymogens other than trypsinogen[73] raises two important questions: a) whether trypsin activation itself, which is clearly cathepsin B-dependent, is directly involved in acinar cell damage and, b) whether a cathepsin B-induced activation of other digestive proteases ultimately causes the pancreatic necrosis for which trypsin is not the culprit. In order to study the role of cathepsin B in the human pancreas tissue specimens and pancreatic juice from patients with hereditary and sporadic pancreatitis were recently investigated. Cathepsin B was clearly shown to be abundantly present in the subcellular secretory compartment of the healthy human pancreas and in the pancreatic juice of controls and pancreatitis patients.[74] It was also found to be a potent activator of human trypsinogen. Its capacity to activate trypsinogen, however, was not affected by the most common trypsinogen mutations found in association with hereditary pancreatitis. While these data indicate that the onset of human pancreatitis may well involve mechanisms that depend on cathepsin B-induced protease activation the cause of hereditary pancreatitis cannot be easily reduced to an altered activation of mutant trypsinogen induced by cathepsin-B.

Role of Trypsin in Premature Digestive Protease Activation

The question of why structural changes in the cationic trypsinogen gene caused by germline mutations would lead to the onset of hereditary pancreatitis has been a matter of debate. Since trypsin is the oldest known digestive enzyme, because

trypsin can activate multiple other digestive proteases in the gut, and because pancreatitis is regarded as a disease caused by proteolytic autodigestion of the pancreas it would be reasonable to assume that pancreatitis is caused by a trypsin-dependent protease cascade within the pancreas itself. If this hypothesis were correct the trypsinogen mutations that are found in association with hereditary pancreatitis could be predicted to confer a gain of enzymatic function,[32,33] meaning that mutant typsinogen would be more readily activated inside acinar cells or, alternatively, active trypsin would be less rapidly degraded inside acinar cells. Both events would lead to a prolonged or increased enzymatic action of trypsin within the cellular environment. In order to prove this hypothesis *in vitro* studies were performed that have analyzed the biochemistry of recombinant human trypsinogens into which pancreatitis-associated mutations were introduced. Some of these studies found, indeed, that either a facilitated trypsinogen autoactivation or an extended trypsin activity can result under defined experimental conditions.[75–77] Whether these *in vitro* conditions reflect the highly compartmentalized situation under which protease activation begins intracellularly and *in vivo*[78,79] is presently unknown, but the above studies would strongly be in favor of a gain of trypsin function as a consequence of defined mutations. A number of arguments, however, have been raised against the gain of trypsin function hypothesis of hereditary pancreatitis. Most hereditary disorders, including many autosomal dominant diseases, are associated with loss-of-function mutations that render a specific protein either defective or impair its intracellular processing and targeting.[80] Moreover, a total of 16 mutations in the cationic trypsinogen gene, scattered over the various regions of the molecule, have been reported to be associated with pancreatitis or hereditary pancreatitis. It is unlikely that such a great number of mutations located in entirely different regions of the PRSS1 gene would all have the same effect on trypsinogen and result in a gain of enzymatic function. A loss of enzymatic function *in vivo* would, accordingly, be a much simpler and consistent explanation for the pathophysiological role of hereditary pancreatitis mutations. This is especially true for the A16V mutation which affects the signal peptide cleavage site involved in the correct processing of trypsinogen.[81] This mutation would not be expected to have an effect on activation or catalytic activity of trypsin. The ultimate proof for the loss of function hypothesis would be a hereditary pancreatitis family in which a significant deletion in the cationic trypsinogen gene, preferably affecting the sequence that encodes the active site, would be found to segregate with the disease phenotype. This piece of evidence, however, is conspicuously missing so far.

In the absence of animal models in which human trypsinogen with a disease-relevant mutation has replaced the wild-type rodent trypsinogens – a daunting task because several genes and pseudogenes need to be exchanged – studies using isolated pancreatic acini and lobules are an alternative to study the role of trypsin in pancreatitis. In one study that used a specific, cell permeant and reversible trypsin inhibitor it was found that complete inhibition of trypsin activity does not prevent, not even reduce, the conversion of trypsinogen to trypsin.[82] A cell permeant cathepsin B inhibitor, on the other hand, prevented trypsin activation completely. In inhibitor wash-out experiments it was determined that, following hormone-induced trypsinogen activation in pancreatic acinar cells, 80% of the active trypsin is immediately and directly inactivated by trypsin itself. Taken together these experiments suggest that trypsin activity is neither required nor involved in trypsinogen activation, that trypsin does not autoactivate in living

pancreatic acinar cell, and that its most prominent role is in autodegradation.[82] This, in turn, suggests that intracellular trypsin activity might have a role in the defense against other, potentially more harmful digestive proteases and that structural alterations that impair the function of trypsin in hereditary pancreatitis would eliminate a protective mechanism rather than generate a triggering event for pancreatitis.[83] Whether these experimental observations obtained from rodent pancreatic acini and lobules have any relevance to human hereditary pancreatitis is presently unknown because human cationic trypsinogen may have different characteristics in terms of its ability to autoactivate and to autodegrade *in vivo*.

Recently reported kindreds with hereditary pancreatitis that carry a novel R122C mutation[84–86] are very interesting in this context. The single nucleotide exchange in these families is located only one 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 recombinant protein was used for biochemical studies, the enterokinase-induced activation, the cathepsin B-induced activation, and the autoactivation of Cys-122 trypsinogen were found to be significantly reduced by 60–70% compared to 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, both of which confer a dramatic loss of trypsin function that cannot be compensated for by increased autoactivation. If this scenario reflects, indeed, the *in vivo* conditions within the pancreas, it would represent the first direct evidence from a human study for a potential protective role of trypsin in pancreatitis.[84] The question of whether the gain of function hypothesis or the loss of function hypothesis correctly predicts the pathophysiology of hereditary pancreatitis can presently not be completely 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 rodent studies, however, suggest that the role of trypsin in the onset of pancreatitis may be more complex than previously assumed and that other proteases are more directly involved in the proteolytic damage during pancreatitis against which trypsin could represents a safeguard.[87]

Role of Calcium in Premature Pancreatic Protease Activation

Calcium is highly concentrated in zymogen granules and has a marked effect on the stability and activation kinetics of protease zymogens in pancreatic juice. Several factors, however, make it difficult to study a direct involvement of calcium in protease activation. First, cleavage of the activation peptide from the N-terminal end of trypsinogen can be catalyzed by enterokinase (enteropeptidase), by lysosomal enzymes such as cathepsin B,[61,62] and by trypsin itself. Furthermore, human trypsinogen, at least under *in vitro* conditions, can auto-activate.[88] To complicate matters further the auto-activation capacity of trypsin varies greatly between trypsin subtypes and among species.[88–90] Once active, trypsin can not only activate trypsinogen but can also degrade and inactivate trypsin, the predominant role of trypsin after intracellular activation within living acinar cells.[82] When a very small amount of active trypsin is added to a purified solution of inactive trypsinogen the

substrate specific activity rapidly increases as trypsinogen is activated. After reaching a maximum activity, however, trypsin-induced trypsin degradation prevails and activity decreases. The role of calcium must be considered in the context of these factors. A number of elegant *in vitro* studies have shown that after trypsinogen activation, calcium stabilizes trypsin.[60,88–90] With regard to auto-activation and auto-degradation, calcium influences both events in a stabilizing manner and thus its presence significantly delays trypsin-induced trypsinogen activation as well as trypsin-induced trypsin-degradation, allowing trypsin activity to persist much longer once activation has occurred.

These *in vitro* mechanisms may be of clinical relevance in a situation where trypsinogen has been secreted from the pancreas but cannot flow freely from the pancreatic duct, e.g. in the event of an obstructing gallstone or tumor,[91] but they may not be applicable to the situation inside the acinar cell.

Ca^{2+} is also a critical intracellular second messenger for the regulated exocytosis of digestive enzymes from the apical pole of the acinar cell. Endocrine diseases that are associated with clinical hypercalcemia are known to predispose patients to develop pancreatitis[92] and those who develop pancreatitis after extracorporeal blood circulation for major cardiac surgery are thought to develop the disease because of an exposure to supraphysiological concentrations of calcium.[93] In animal experiments hypercalcemia was shown to either decrease the threshold level for the onset of pancreatitis or to induce morphological alterations equivalent to pancreatitis.[92,94] Moreover, in studies that have investigated the initial phase of experimental pancreatitis a progressive disruption of the intracellular Ca^{2+}-signaling was reported.[24] It has therefore been proposed that an elevation of acinar cytosolic free ionized calcium should be regarded as the most probable common denominator for the onset of various clinical varieties of acute or chronic pancreatitis.[95] Recent studies, in which the effect of a disruption of intracellular Ca^{2+}-signaling on premature protease activation in isolated acini was studied seem to confirm this hypothesis. Regardless of whether intracellular Ca^{2+}-stores were depleted by calcium-ATP-ase inhibition, withdrawal of extracellular Ca^{2+}, or complex formation with Ca^{2+}-chelators, intracellular protease activation in response to supramaximal hormone stimulation was greatly reduced or abolished[78,96]. However, increasing intracellular Ca^{2+}-concentrations with Ca^{2+}-ionophores or the Calcium ATPase inhibitor Thapsigargin did not induce premature protease activation. These experiments indicate that high intracellular Ca^{2+} concentrations are a requirement for premature protease activation but may not be sufficient to induce this process. While the requirement for calcium in protease activation is now undisputed some authors believe that elevated intracellular calcium in general, and regardless of its subcellular site and mechanism of release, is sufficient to trigger premature protease activation.[97] The latter view remains in conflict with trials that used other lines of evidence in addition to single cell measurements.[78,96] While all of the above studies used hormone-induced models of intra-acinar cell protease acitivation the most recent investigation could demonstrate that changes in intracellular calcium dynamics are also involved in the onset of pancreatitis in models that mimic the human disease.[18]

Conclusions

Recent advances in cell biological and molecular 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 pancreatic research. 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 for the prevention and cure of this debilitating disease.

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