Pathobiology of Experimental Acute Pancreatitis

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Pancreatic duct obstruction, even in the absence of biliary obstruction and/or bile reflux into the pancreatic duct, can trigger acute hemorrhagic necrotizing pancreatitis. The earliest changes are seen within acinar cells. Early derangements in acinar cell biology include inhibition of digestive enzyme secretion and the co-localization of lysosomal hydrolases with digestive enzyme zymogens. Under appropriate conditions, this co-localization could lead to digestive enzyme activation within acinar cells.

INTRODUCTION

Acute pancreatitis is an inflammatory disease of the pancreas, which is associated with a number of other disease processes that are, collectively, known as the "etiologies" of pancreatitis. Included among these etiologies of acute pancreatitis are a number of processes such as trauma to the pancreas, exposure to certain drugs or toxins, abnormalities of plasma lipoproteins, and certain infections. The vast majority of patients with acute pancreatitis, however, develop their disease either in conjunction with the abuse of alcohol or, more commonly, in association with biliary tract stone disease. The studies of Acosta and Ledesma [1] as well as those of others have clearly indicated that the acute pancreatitis which occurs in association with biliary tract stone disease is precipitated by the passage of stones into or through the terminal biliary ductal system. When Acosta and Ledesma screened the stools of patients with biliary tract stone disease who either did or did not have associated pancreatitis, they noted the presence of gallstones in the feces of 90 percent of patients with acute pancreatitis but only 11 percent of patients who were not experiencing an attack of acute pancreatitis.

The events which couple passage of biliary tract stones into or through the terminal biliary ductal system and the development of an acute inflammatory reaction within the pancreas are not well understood. In this paper, I review the results of studies which we have performed over the past decade in an attempt to elucidate some of those mechanisms. We have attempted to address three areas of uncertainty. First, we have sought to determine whether the stone which triggers the attack of acute pancreatitis does so by causing bile reflux into the pancreatic ductal system or, alternatively, whether that stone triggers acute pancreatitis by obstructing the pancreatic duct. Second, we have attempted to define where, within the pancreas, the earliest lesion of pancreatitis develops. Third, we have tried to elucidate the intracellular events which might precipitate acute pancreatitis by causing digestive enzyme activation within the pancreas.

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Abbreviation: CCK: cholecystokinin

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THE QUESTION OF BILE REFLUX

Three theories have been proposed which might explain the mechanism by which passage of a stone through the distal biliary ductal system might trigger acute pancreatitis. The first was that proposed by Opie in 1901, when he reported several patients who, at autopsy after death from acute pancreatitis, were noted to have one or more stones impacted in the distal biliary ductal system [2]. Opie noted that, in those patients, the obstructing stone had created behind it a "common channel" which allowed communication between the biliary and the pancreatic ductal systems, and he reasoned that the distal obstruction might permit bile to reflux into the pancreatic ductal system through this common channel. He suggested that bile, refluxed into the pancreatic ductal system, would have a noxious effect on the pancreas and precipitate acute pancreatitis. This so-called "common channel theory" has been the subject of extensive investigation since that time, but its validity remains controversial.

A second theory, the so-called "duodenal reflux theory," suggests that the offending stone is passed through the sphincter of Oddi and, as a result of stone passage, the sphincter is stretched and made incompetent. Subsequent reflux of duodenal contents containing activated digestive enzymes from the duodenum into the pancreatic ductal system through the incompetent sphincter might, according to this theory, precipitate acute pancreatitis. This duodenal reflux theory has, in general, been discounted as the result of the clinical observation that patients undergoing either surgical or endoscopic sphincter ablative procedures do not routinely develop acute pancreatitis.

A third theory has been proposed which suggests that the offending stone or, alternatively, the edema and inflammation caused by stone passage, results in obstruction of the pancreatic ductal system. It is believed that, with continued secretion into the obstructed duct, ductal hypertension could result, and, by as yet unidentified mechanisms, this process might lead to acute pancreatitis.

Since Opie's original description of the common channel theory in 1901, many studies have clearly demonstrated that bile, injected under pressure into the pancreatic ductal system, can cause pancreatic injury. It has remained unclear, however, whether it is the bile which precipitates pancreatitis under these conditions or, alternatively, the pressure of the injection which precipitates the attack. The importance of ductal hypertension and ductal obstruction as triggering events in pancreatitis has been difficult to define since, for the most part, animals subjected to pancreatic duct ligation develop pancreatic atrophy rather than acute inflammation within the pancreas.

Recently, it has been noted that hemorrhagic necrotizing pancreatitis can be induced in the American opossum by ligation of the terminal biliary-pancreatic ductal system of that animal [3,4]. The American opossum has a very long extra duodenal common biliary-pancreatic duct as well as an easily identifiable main pancreatic ductal system. We have carried out a number of investigations, using this experimental model of pancreatitis, to determine whether or not bile reflux is important in the development of acute pancreatitis. In these studies, four groups of animals were prepared. In addition to a control or unoperated group, some animals underwent ligation of the common biliary-pancreatic duct immediately adjacent to its point of entry into the duodenum. Because of the long common biliary-pancreatic channel that the opossum has, this group of animals were exposed to pancreatic
outflow obstruction under conditions which permitted bile reflux into the pancreatic ductal system. Another group of animals underwent separate ligation of the bile duct and the pancreatic duct. As a result, these animals experienced pancreatic ductal outflow obstruction as well as extrahepatic biliary ductal obstruction under conditions which prevented bile reflux into the pancreatic ductal system. Another group of animals underwent ligation of the pancreatic duct alone without accompanying bile duct obstruction. Finally, a jaundiced control group of animals were prepared, in which only the bile duct was obstructed.

Hemorrhagic necrotizing pancreatitis developed in the animals subjected to pancreatic duct obstruction with, as well as without, accompanying bile duct obstruction. In addition, hemorrhagic necrotizing pancreatitis developed in the animals undergoing ligation of the combined biliary-pancreatic duct adjacent to the duodenum. When careful morphometric examination of the severity of pancreatitis was carried out, we noted that the severity of pancreatitis was similar in each of these three groups.

We concluded, from these studies, that pancreatic duct obstruction is, by itself, sufficient to induce severe necrotizing hemorrhagic pancreatitis in the American opossum. The presence of accompanying bile duct obstruction does not appear to worsen the severity of pancreatitis in these animals. Potentially more significant, however, was our observation that ligation of the common biliary pancreatic ductal system of the opossum, a procedure which causes pancreatic duct obstruction while permitting bile reflux into the pancreas, does not result in pancreatitis that is more severe than that noted after isolated pancreatic duct obstruction under conditions which prevent bile reflux. This observation has led us to conclude (a) that bile reflux is not required for the development of acute necrotizing pancreatitis and (b) that bile reflux does not worsen the acute pancreatitis which follows pancreatic duct obstruction [5].

LOCATION OF THE EARLIEST LESIONS

The location of the earliest lesions of pancreatitis has been the subject of considerable controversy. Studies of early pancreatitis in humans have been unable to answer this question because the clinical disease is usually far advanced before the pancreas can be examined morphologically. Kloppel et al. have suggested that the earliest lesions of pancreatitis develop in the peripancreatic connective tissue and in the perilobular regions of the pancreas [6]. In contrast, Foulis [7] has argued that acute pancreatitis develops in the periductal area of the pancreas. In order to explore this question further, we have studied the very earliest events which occur after ligation of the pancreatic ductal system of the opossum. As noted above, ligation of the ductal system in the opossum leads to severe hemorrhagic necrotizing pancreatitis. Gross changes are noted within three days of duct ligation in that animal and, by 14 days, most of the animals have died of acute necrotizing pancreatitis. We studied a group of animals which had been subjected to combined biliary-pancreatic ductal obstruction for three, six, 12, and 24 hours. We noted that acinar cell necrosis could be detected within three hours of duct ligation and that areas of necrosis increased in size over the ensuing 12 hours. Widespread patchy areas of acinar necrosis could easily be detected microscopically within 12 hours of duct ligation, yet, at that time, no evidence of either periductal or perilobular inflammation was found. By 24 hours following duct ligation, early changes of periductal inflammation could be seen. We
concluded, from these studies, that the earliest changes in this model of acute pancreatitis occur within the acinar cells themselves and that neither periductal nor perilobular changes were, in fact, the earliest lesions of the disease [8].

PREMATURE DIGESTIVE ENZYME ACTIVATION

There are several pieces of circumstantial evidence which have suggested that acute pancreatitis results from autodigestion of the gland by enzymes which are normally synthesized and secreted by the pancreas. That evidence includes the fact that the pancreas does, indeed, synthesize a large amount of digestive enzyme protein. In addition, during acute clinical pancreatitis, activated forms of digestive enzymes have been detected within the pancreas itself. Finally, the morphological changes of severe acute pancreatitis suggest that a coagulation necrosis or digestive process has occurred. Although this evidence has suggested that pancreatitis may involve digestion of the gland parenchyma, the mechanisms responsible for that process have evaded elucidation. Indeed, the pancreas does synthesize a large amount of protein, and the vast majority of that protein is composed of digestive enzymes that are secreted from the acinar cells of the pancreas. Most of those digestive enzymes, however, are synthesized and secreted as inactive proenzymes or zymogens, with normal activation occurring only after they reach the duodenum, where the brush border enzyme enterokinase (enteropeptidase) activates trypsinogen, and trypsin activates the remaining digestive enzymes. Thus, an explanation of the mechanisms leading to digestion of the pancreas during acute pancreatitis must include a mechanism whereby those digestive enzymes might become activated within the gland itself.

*Experimental Models*

In order to study the early events of acute pancreatitis, we have taken advantage of three recently developed models of the disease. The first model is that developed by Lombardi and co-workers [9], who noted that feeding young female mice a diet which was choline-deficient yet enriched in ethionine (the CDE diet) could cause hemorrhagic pancreatic necrosis which, if the diet was fed continuously, was uniformly fatal within five days. For our own studies, we have modified the protocol originally developed by Lombardi and co-workers so as to induce a slightly more mild disease and one which would be easily manipulable during the early stages. We have used young female mice weighing between ten and 14 grams and fasted those mice for 24 hours prior to exposing them to the CDE diet. After being fed three grams of the diet per mouse over a 24-hour period, the mice are once again fasted for 24 hours and then placed back on normal laboratory chow. Using this protocol, a five-day mortality rate of between 50 and 70 percent can be attained. Little or no evidence of gross injury to the pancreas can be detected within the first two or three days of this experimental protocol.

We have also employed a model of pancreatitis which is less severe than that induced by the choline-deficient, ethionine-supplemented diet. That model, originally developed by Lampel and Kern [10], involves infusing rats with a dose of the CCK analog caerulein in excess of that dose which elicits a maximal rate of digestive enzyme secretion. This so-called "supramaximal stimulation" elicits a transient, reversible, and non-fatal form of edematous pancreatitis. Edema can be noted within
one hour of the beginning of the infusion, and, in general, the lesion regresses over the subsequent several days after cessation of the infusion.

The final model that we have employed represents an attempt to duplicate the events that follow pancreatic duct obstruction. We have used both rats and rabbits for these experiments. To duplicate ductal obstruction, we have cannulated the pancreatic duct and subsequently elevated the indwelling cannula to a vertical position so that the animal secretes juice into the cannula until the hydrostatic pressure of the column of secreted juice matches the secretory pressure of the gland. At that time, the duct is functionally obstructed [11].

We have noted that each of these three models, although strikingly different in terms of the actual manipulations occurring and the severity of pancreatitis which results, brings about a remarkably similar constellation of changes. All three models cause demonstrable pancreatic edema as well as marked hyperamylasemia. In our subsequent studies, we have attempted to identify other features which might be common to all three of these models in the belief that those events that might be common in such otherwise dissimilar models of pancreatitis might also be important in the clinical disease. We have focused our attention on the events related to protein and digestive enzyme synthesis as well as intracellular transport within the pancreas because of the circumstantial evidence mentioned above, which has suggested that acute pancreatitis might result from autodigestion of the gland.

*Digestive Enzyme Synthesis and Secretion*

Our initial studies focused on the processes of digestive enzyme synthesis and secretion from the pancreas. The acinar cell of the pancreas is the most active protein-synthesizing cell in the body and over 90 percent of the protein synthesized by the pancreas is digestive enzyme protein destined to be exported from the cell. We have employed the pulse label technique to evaluate the processes of synthesis and secretion. The animal was given a bolus of radioactive amino acid and, at selected subsequent times, the pancreas was removed, homogenized, and the incorporation of radioactivity into protein measured. The rate of appearance of radioactivity labeled protein is, using this technique, a measure of the rate and extent of protein synthesis. In the normal pancreas, radioactively labeled protein accumulated with time until a maximal level was reached, and, subsequently, radioactivity declined as the newly synthesized protein was exported from acinar cells, transported through the pancreatic ductal system, and discharged into the duodenum. On the other hand, in all three of the models of pancreatitis (diet-induced, secretagogue-induced, and duct obstruction-induced), although digestive enzyme synthesis was unaffected, the process of digestive enzyme secretion was markedly reduced. We have concluded from these studies that an early event in the evolution of pancreatitis involves the blockade of digestive enzyme secretion from acinar cells [11–13].

*Intracellular Trafficking*

Proteins are assembled within acinar cells on the rough endoplasmic reticulum. The nascent polypeptide chains elongate within the cisternae of the endoplasmic reticulum, and, after synthesis has been completed, the newly synthesized proteins are transported to the Golgi complex. Most of the current evidence indicates that protein sorting occurs primarily in the Golgi complex. Some enzymes, such as those destined to be transported to lysosomes, are glycosylated in the Golgi complex, while
others undergo other post-translational modifications. Proteins such as digestive enzymes, which are destined to be exported from the cell, are believed to pass through the Golgi complex and are packaged in zymogen granules which mature into condensing vacuoles as they move to the luminal surface of the cell. At the luminal surface, and in response to hormonal stimulation, the limiting membrane of the zymogen granule fuses with the luminal plasma membrane, and, as a result, the contents of the zymogen granule are discharged into the extracellular ductal space [14]. Subsequent to their being glycosylated, lysosomal enzymes are modified by phosphorylation at the 6 position of mannose residues. The 6-mannose-phosphorylated proteins are bound by mannose-6-phosphate-specific receptors in the Golgi, which then shuttle between the Golgi complex and the pre-lysosomal compartment, transporting lysosomal enzymes away from the secretory pathway and into the lysosomal compartment [15].

We were particularly interested in the possibility that a defect in sorting of digestive enzymes and lysosomal enzymes might occur during the early stages of pancreatitis because of previously reported evidence that the lysosomal enzyme cathepsin B could, under certain conditions, cause the activation of trypsinogen [16,17]. Since trypsin is capable of activating the remainingzymogens, intracellular activation of trypsinogen by cathepsin B could eventually lead to activation of most of the digestive enzymes.

In order to evaluate possible changes in the intracellular trafficking of digestive enzymes and lysosomal enzymes, we have employed the techniques of subcellular fractionation as well as immunolocalization [18–21]. In our subcellular fractionation studies, the pancreas was removed, homogenized, and then subjected to varying degrees of centrifugal force. As a result, a high-density zymogen granule pellet was obtained, a somewhat lower-density pellet enriched in lysosomes and mitochondria was obtained, and, finally, a microsomal as well as a soluble fraction was obtained after high-speed centrifugation. As expected, most of the digestive enzyme protein was recovered in the zymogen granule fraction, and more than 50 percent of the lysosomal enzyme activity was detected in the lysosome-mitochondrial fraction. Approximately 20 to 25 percent of the lysosomal enzyme activity was also recovered in the zymogen granule fraction under normal conditions, a phenomenon which was initially interpreted as indicating the presence of relatively high-density lysosomes. More recent studies, however, have indicated that a significant fraction of lysosomal enzymes normally escape complete sorting in the Golgi complex and, as a result, that fraction of lysosomal enzymes is packaged in the secretory vesicles of the secretory pathway [22]. We have noted that there is a marked increase in the amount of lysosomal enzyme which is recovered in the zymogen granule fraction during the early stages of all three models of experimental pancreatitis, and, at the same time, there is a corresponding reduction in the amount of lysosomal enzyme recovered in the lysosomal fraction in those models. This observation strongly suggests that mis-sorting of lysosomal enzymes might occur during the early stages of experimental pancreatitis and that, as a result, lysosomal enzymes and digestive enzymes might become co-localized within the same intracellular organelles. Other interpretations of the results of subcellular fractionation experiments are certainly possible, however, including the fact that the enzymes might be recovered within the same fraction yet not occupy the same organelle. To complement our subcellular fractionation studies, therefore, we have also employed morphological techniques of immunolocal-
Mechanistic Studies

Acinar cells of the pancreas normally secrete their stored digestive enzymes in response to neurohumoral stimulation. The neurotransmitter acetylcholine and the hormone cholecystokinin (CCK) bind to cell surface receptors and trigger intracellular events that result in the discharge of stored digestive enzymes into the ductal space. This process, collectively known as stimulus-secretion coupling, involves a number of events that occur in sequence. Subsequent to hormone receptor binding, activation of the membrane-bound enzyme phospholipase C occurs in a GTP-dependent manner and, as a result, membrane phospholipids such as PIP2 are hydrolyzed. The products of PIP2 hydrolysis are IP3 and diacylglycerol. They act as intracellular messengers to stimulate the release of calcium from intracellular stores and to activate protein kinase C. We have evaluated the changes in stimulus-secretion coupling which occur during the early stages of diet-induced and secretagogue-induced pancreatitis. In the diet-induced model, we found that stimulus-secretion coupling is blocked at the level of phospholipase activation; that is, hormone receptor binding remains normal, yet exposure to hormone fails to elicit the generation of IP3 or release of intracellular calcium [24]. Secretion can, under these conditions, be restored if the block is bypassed by direct stimulation with a calcium ionophore or by administration of a diacylglycerol analog or a phorbol ester. These observations have suggested that the ethionine-containing diet, by as yet undefined mechanisms, prevents the activation of phospholipase C, which normally follows
hormone receptor occupancy. This block might, possibly, involve an alteration of GTP-binding protein activity.

The blockade of secretion which occurs after supramaximal stimulation with caerulein appears to involve another mechanism. Caerulein, as noted previously, is a CCK analog, and, as a result, it can be expected to bind to cell surface CCK receptors. Pancreatic acinar cells are believed to have both high-affinity and low-affinity CCK receptors, with the former mediating the process of digestive enzyme secretion, while the latter appear to have an inhibitory role in this process. Recently, a CCK analog known as CCK-JMV-180 has been developed [25], which acts as an agonist at the high-affinity stimulatory CCK receptors and as an antagonist at the low-affinity inhibitory CCK receptors. We have evaluated the ability of this CCK analog to induce pancreatitis and its effect on pancreatitis induced by caerulein. Those studies were based on the prediction that, if pancreatitis induced by caerulein was mediated by the high-affinity receptors, than the CCK-JMV-180 analog would induce pancreatitis itself. On the other hand, if secretagogue-induced pancreatitis was mediated by the low-affinity inhibitory receptors, we would predict that the CCK-JMV-180 analog would not induce pancreatitis, but that it would inhibit pancreatitis induced by caerulein. We have found that, indeed, CCK-JMV-180 is incapable of inducing pancreatitis and that the analog prevents pancreatitis induced by high doses of caerulein. As a result, we have concluded that secretagogue-induced pancreatitis is mediated by the low-affinity inhibitory CCK receptors [26].

The mechanisms by which low-affinity receptor occupancy might result in a defect in secretion as well as intracellular co-localization of digestive enzymes and lysosomal hydrolases is not entirely clear. Since pancreatic edema, hyperamylasemia, and subcellular enzyme redistribution can be detected within one or two hours of supramaximal in vitro stimulation, we reasoned that similar events might be detected after exposing acinar cells to in vitro supramaximal stimulation. Clearly, hyperamylasemia and pancreatic edema cannot be measured in a system which involves in vitro incubation of acinar cells with supramaximally stimulating doses of hormone. On the other hand, the phenomenon of lysosomal enzyme redistribution is easily measured under those conditions, and, we postulated, might be a useful method of following the evolution of this "in vitro model" of acute pancreatitis. We were surprised to note, however, that exposure of acini to supramaximally stimulating concentrations of caerulein did not result in subcellular lysosomal enzyme redistribution. When acini were incubated with a supramaximally stimulating concentration of caerulein along with plasma obtained from an animal infused with a supramaximally stimulating dose of caerulein, however, subcellular lysosomal enzyme redistribution was, in fact, noted. This observation suggests that in vivo supramaximal stimulation results in the elaboration of a plasma factor which can, under appropriate conditions, result in the in vitro subcellular redistribution of lysosomal enzymes. We have fractionated the plasma used in these experiments and noted that the active factor is a protein with a molecular weight between 10,000 and 30,000 daltons and that this protein appears to have proteolytic enzyme activity. A similar plasma factor was also detected in samples obtained from opossums several days after duct obstruction. Taken together, these observations have suggested that a plasma factor may be elaborated during the early stages of pancreatitis and that this plasma factor may play an important role in causing the intracellular biochemical changes that eventually lead to digestive enzyme activation [27].
FUTURE DIRECTIONS

There are a large number of questions which remain unanswered regarding the pathogenesis and evolution of pancreatitis, both from a clinical as well as an experimental standpoint. For example, the factors which determine the severity of pancreatitis remain completely unknown. The models that we have used result in markedly different severities of pancreatitis. The diet-induced model results in massive hemorrhagic pancreatic necrosis with death of the animal, whereas secretagogue-induced pancreatitis is a transient and mild form of the disease, characterized primarily by edema and hyperamylasemia. Duct obstruction in rats and rabbits leads to transient pancreatic edema but little evidence of inflammation, whereas duct obstruction in the opossum appears to lead to hemorrhagic necrosis of the gland with death of the animal. In spite of these marked differences in severity and outcome, most of the events which we have characterized were found to occur in all of these models of pancreatitis. Clearly, other events must be superimposed on those which we have identified, and those additional events must be important determinants of the severity of pancreatitis. It would be extremely important and clinically useful to identify those events, since it is those processes which might be the target of the therapeutic interventions. Another important area of uncertainty involves the time-dependence of pancreatitis. It is as yet unclear whether the severity of clinical pancreatitis is determined at the moment of its onset or, alternatively, whether it is determined by the duration of pancreatic duct obstruction. From a clinical standpoint, the timing and choice of therapy depend on resolution of this question. Some have advocated early operative or endoscopic intervention to remove potentially obstructing stones, whereas others have concluded that the offending stone has, most likely, passed by the time the patient presents to the hospital and that, in any case, the severity of pancreatitis has already been determined. Thus, resolution of this question may have important implications on the therapeutic approach employed. It is hoped that studies employing the models described above can answer some of these remaining critically important questions.

REFERENCES