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Bile Duct Response to Repeated Cerulein Induced Pancreatitis in the Rat

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Abstract: The typical morphology of common bile duct strictures in chronic pancreatitis suggests a pathogenesis not of encasement, but of mural changes in the common bile duct. Cerulein, 20 µg/kg was administered to male Wistar rats in four subcutaneous doses, hourly for 3 h, and was recorded as one application. Group A served as controls and received subcutaneous saline. Group B1 received one application of Cerulein and was sacrificed after 24 h. Group B2 was similar to B1, but was sacrificed two weeks later. Group B3 was given two applications of Cerulein with an interval of two weeks and was sacrificed two weeks after the second application. Group B4 received one application of Cerulein every week for four weeks and was sacrificed two weeks after the last application. Serum amylase levels, pancreatic edema and peripancreatic inflammatory infiltration scores increased with the severity and persistance of the insult. Bilirubin and alkaline phosphatase levels suggested cholestatic response in the groups that received repeated insults with Cerulein (Groups B3 and B4; $p < 0.01$). Moderate to strong immuno-histochemical positivity for type IV collagen at the wall of the common bile duct in animals with a persisting insult is suggestive of circumstantial evidence for mural changes as a causative factor of stricture formation in similar settings such as chronic pancreatitis with persistent inflammatory attacks.

Key Words: Pancreatitis, chemically induced; Pancreatitis, complications; Bile duct strictures; Rat pancreatitis.

Introduction

Extrahepatic cholestasis is a well known complication of chronic pancreatitis (1-3). Although only 3 to 8% of patients experience persistent jaundice, cholestasis in the course of the disease may be seen in 27 to 45% of the patients in various series (4-6). The cholestasis of chronic pancreatitis may be partly induced by the edema and inflammation accompanying the disease, as the distal common bile duct is enveloped in the pancreatic head in 75% of the human population (7). However, in most patients presenting with persistent jaundice due to extrahepatic biliary obstruction, the fundamental disorder is attributed to the compression and/or infiltration of the biliary passage by fibrosis associated with chronic pancreatitis. This acceptance has led to surgical procedures that relieve jaundice with local resections or resections that preserve the duodenum and even the integrity of the common bile duct (8). Hence, there is reasonable evidence that extrahepatic jaundice in chronic pancreatitis is due to encasement of the common bile duct by the surrounding pancreas. However, there is no evidence that excludes the possibility of intramural fibrosis in the common bile duct, resulting from repeated inflammatory attacks during the course of this chronic process. In this study, we examined the effects of repeated inflammatory attacks of the pancreas on the structural changes of the common bile duct in a rat model.

Materials and Methods

Cerulein, at a dose of 20 µg/kg, was administered to male Wistar albino rats, six weeks of age, weighing 120 to 180 g. The total dose of Cerulein was delivered in four hourly subcutaneous injections, recorded as one application. The rats were divided into five groups. Group A (n = 3) served as controls and received one application of isotonic saline subcutaneously. Group B1 (n = 5) rats received one application of Cerulein and were sacrificed 24 h after the last injection. Group B2 rats (n = 7) received one application of Cerulein, and were sacrificed two weeks later. Group B3 rats (n = 7) were given two applications of Cerulein at an interval of two weeks and were sacrificed two weeks after the last application of the drug. Group B4 rats (n = 7) received one application of

Cerulein every week for four weeks and were sacrificed two weeks after the last application.

The animals were fed commercially available standard chew and had free access to water throughout the protocol. The rats were sacrificed by guillotine decapitation under ether anesthesia. Blood samples were analyzed for ALT, AST, amylase, alkaline phosphatase, and total and direct bilirubin using an Express 550 Ciba-Corning Autoanalyzer. The abdomen was swiftly opened and the liver, bile ducts and pancreas were removed en-bloc for histopathological examination.

A light microscopic examination was performed after the tissue samples were dyed with hematoxylin and eosin. Gomori's methenamine silver dye method and Masson's trichrome method were used to assess the connective tissue response. All tissue samples were examined immediately after the respective group of rats were sacrificed; the pathologist was blind to the groups. A scoring system was created to enable comparison between the groups. Pancreatic, peripancreatic and choledochal cellular inflammatory changes were graded as shown in Table 1.

Mural fibrinogen, laminin and type IV collagen were examined immunohistochemically as predictors of fibrosis of the common bile duct. Their presence was evaluated by using Dako Laboratories (Carpinteria, CA, USA) monoclonal Mouse Anti-Human Collagen IV, Rabbit Anti-Human Fibrogen, and Monoclonal Mouse Anti-Human Laminin primary antibodies; with Universal LSAB Kit, AEC Substrate-Cromogen and water soluble Harris Hematoxylin for counterstaining. A separate scoring system (Table 2) was used to evaluate the immunohistochemical results.

The results of biochemical studies were analyzed by Student's t-test. The Kruskal-Wallis test was employed to detect differences in histopathological and immunohistochemical results between the groups. Whenever a difference was detected by the Kruskal-Wallis test, the Mann-Whitney Rank Sum test was used to explore significance between the groups. At least $p = 0.05$ was regarded as statistically significant.

Results

The biochemical results for all groups are shown as mean (± SEM) values in Table 3. The serum amylase was 680 (92.7) U/L for Group A controls, 1350 (24.7) U/L

Table 1. Scoring for Histopathological Evaluation.

Pancreatic:	
Edema:	
0	Absent
1	Mild
2	Moderate
3	Significant
Inflammatory Cellular Infiltration (Polymorphonuclear and mononuclear):	
0	No cellular infiltration
1	Homogeneous cellular infiltration
2	Cluster-like cellular infiltration
Peripancreatic Inflammatory Cellular Infiltration:	
0	Groups of mononuclear cells within fat tissue, with normal pancreatic architecture
1	Two-fold increase in mononuclear cells in peripancreatic fat
2	Inflammatory infiltration including polymorphonuclear leucocyte within peripancreatic fatty tissue
Choledochal:	
Edema:	
0	Absent
1	Mild
2	Significant
Cellular Infiltration:	
0	No polymorphonuclear cellular infiltration at the wall of the CBD
1	Mild polymorphonuclear cell infiltrates at the wall of the CBD
2	Significant polymorphonuclear cellular infiltration
Mural Connective Tissue:	
0	No increase in the amount of connective tissue or reticulum fibers
1	Increase in the amount of connective tissue and reticulum fibers.

Table 2. Scoring for Immunohistochemical Evaluation.

Common Bile Duct Wall Fibrinogen:	
0	Negative
1	Mild positive
2	Moderate positive
Common Bile Duct Wall Laminin Content:	
0	Negative
1	Mild positive
2	Moderate positive
Common Bile Duct Wall Type IV Collagen Content:	
0	Negative
1	Mild positive
2	Moderate positive
3	Strong positive

Table 3. Biochemical results for all groups. The figures are means (±SEM). All results are in U/L, except bilirubin (mg/dL).

	Group A	Group B1	Group B2	Group B3	Group B4
Amylase	680 (±92.7)	1350[1] (±24.7)	712 (±57.6)	2464[1] (±110)	2537[1] (±52.2)
Alkaline Phosph.	86 (±13.9)	67.6 (±10.0)	93.9 (±8.8)	316.7[2] (±21.8)	424.3[2] (±20.2)
ALT	94.3 (±6.2)	97.6 (±9.6)	102.14 (±5. 2)	206.0[3] (±15.2)	197.0[3,4] (±14.6)
AST	63.3 (±7.5)	67.2 (±5.6)	72.8 (±3.6)	86.4[5] (±4.4)	93.1[5] (±1.2)
Bilirubin	0.8 (±0.2)	1.3 (±0.2)	1.5 (±0.2)	3.1[6] (±0.4)	4.0[6,7] (±0.1)

[1] Statistically significant compared to Group A (B1, $p < 0.05$; B3, $p < 0.01$; B4, $p < 0.01$). [2] Significant compared to Group A (B3, $p < 0.01$; B4, $p < 0.01$), to Group B1 (B3, $p < 0.05$; B4, $p < 0.005$) and to Group B2 (B3, $p < 0.001$; B4, $p < 0.001$). [3] Significant, compared to Group A (B3, $p < 0.01$; B4, $p < 0.01$) and to Group B1 (B3, $p < 0.005$; B4, $p < 0.005$). [4] Significant, compared to Group B2 ($p < 0.001$). [5] Significant, compared to Group A (B3, $p < 0.05$; B4, $p < 0.01$), to Group B1 (B3, $p < 0.05$; B4, $p < 0.05$), and to Group B2 (B3, $p < 0.05$; B4, $p < 0.01$). [6] Significant compared to Group A (B3, $p < 0.01$; B4, $p < 0.01$), to Group B1 (B3, $p < 0.005$; B4, $p < 0.005$) and to Group B2 (B3, $p < 0.001$; B4, $p < 0.001$). [7] Significant compared to Group B3 ($p < 0.05$).

for Group B1, 712 (57.6) U/L for Group B2, 2464 (110) U/L for Group B3 and 2537 (52.2) U/L for Group B4. Compared to the controls, the serum amylase increased significantly in B1 ($p < 0.05$), B3 ($p < 0.01$) and B4 ($p < 0.01$). There were also significant differences between Groups B1 and B2 ($p < 0.005$), B2 and B3 ($p < 0.001$) and B2 and B4 ($p < 0.001$), indicating that the increase in the cerulein insult produced more marked hyperamylasemia.

Similarly, both alkaline phosphatase and serum bilirubin exhibited steady increases parallel to the increase in the persistence of the Cerulein insult. Alkaline phosphatase was 86 (13.9) U/L in the controls; however, this rose to 316.7 (21.7) U/L in B3 ($p < 0.01$) and 424.3 (20.1) U/L in B4 ($p < 0.01$). There were also statistically significant differences between B1 versus B3 ($p < 0.05$) and B4 ($p < 0.005$), B2 versus B3 ($p < 0.001$) and B4 ($p < 0.001$), and between B3 and B4 ($p < 0.01$). The bilirubin values were 0.83 (0.24) mg/dL for Group A, 1.28 (0.18) mg/dL for B1, 1.48 (0.16) md/dL for B2, 3.14 (0.35) mg/dL for B3 and 4.0 (0.15) mg/dL for B4. The increase was significant between Groups A and B3 ($p < 0.01$), A and B4 ($p < 0.01$), B1 and B3 ($p < 0.005$), B1 and B4 ($p < 0.005$), B2 and B3 ($p < 0.001$), and B2 and B4 ($p < 0.001$). There was also a significant increase between Groups B3 and B4 ($p < 0.05$). These results suggest that the bile flow is disturbed as the pancreatic inflammatory insult is increased.

Serum ALT and AST values were also in accord with the general biochemical response to the model employed. They both revealed statistically significant increases in Groups A to B4 (Table 3).

Light microscopic and immunohistochemical results are shown in Tables 4 and 5, respectively. The results are expressed as the percentage of the animals in the group that exhibited the parameter. Statistical evaluation revealed significance only for light microscopic data for pancreatic edema and peripancreatic inflammatory infiltration. Pancreatic edema was significant between Groups A and B1 ($p = 0.037$), A and B4 ($p = 0.05$), B1 and B3 ($p = 0.018$), and B1 and B4 ($p = 0.008$). Peripancreatic inflammatory cellular infiltration was significantly different between Groups B1 and B3 ($p = 0.05$), B2 and B4 ($p = 0.02$), and B3 and B4 ($p = 0.005$). All other differences between the groups were insignificant.

Discussion

Choledochal strictures in chronic pancreatitis are generally attributed to periductal fibrosis from long standing disease (9). It is believed that when periductal inflammation progresses to fibrosis a fixed stricture results (10) . This belief is a direct extention of the assumption that the pathologic characteristic is encasement of the common bile duct within the pancreas or in its immediate vicinity (11). However, the typical choledochal stricture of chronic pancreatitis is characteristically a long, smooth, gradual tapering of the distal common bile duct (3,10,11). This stricture

Table 4. The results of histopathological evaluation.

Groups	A	B1	B2	B3	B4
Inflammatory Cellular Infiltration					
Homogeneous cellular infiltration	66.7%	60.0%	42.9%	57.2%	5.7%
Cluster-like cellular infiltration	-	-	14.3%	42.9%	14.3%
Pancreatic Edema					
Mild	33.0%	100%	42.9%	-	-
Moderate	-	-	14.3%	85.7%	28.6%
Significant	-	-	-	14.7%	71.4%
Peripancreatic Inflam. Infiltration					
Groups of mononuclears in fatty tissue	66.7%	100%	57.1%	85.7%	14.3%
Two-fold increase in mononuclears	33.3%	-	42.0%	14.7%	28.6%
Polymorphonuclears in inflam. infiltrate	-	-	-	-	57.1%
Common Bile Duct (CBD) Edema					
Mild	-	100%	57.1%	42.9%	42.9%
Significant	-	-	-	14.3%	57.1%
CBD Cellular Infiltration					
Mild polymorphonuclear infiltration	-	80.0%	71.4%	71.4%	71.4%
Significant polymorphonucl. infiltration	-	-	-	14.3%	28.6%
CBD Connective Tissue and Reticulum Fiber					
No increase	100%	100%	100%	100%	100%
Increased	-	-	-	-	-

Table 5. The results of immunohistochemical parameters at the common bile duct wall.

Groups	A	B1	B2	B3	B4
Fibrinogen					
Negative	100%	100%	100%	100%	100%
Mild positive	-	-	-	-	-
Moderate positive	-	-	-	-	-
Laminin					
Negative	1/3*	20.0%	-	14.3%	-
Mild positive	1/3	40.0%	85.7%	71.4%	57.1%
Moderate positive	1/3	40.0%	14.3%	14.3%	42.9%
Type IV Collagen					
Negative	66.7%	80.0%	-	42.9%	57.1%
Mild positive	33.3%	20.0%	71.4%	14.3%	-
Moderate positive	-	-	28.6%	42.9%	28.6%
Strong positive	-	-	-	-	14. 3%

* Group A (normal controls) consisted of three rats.

morphology casts doubt on the encasement theory, especially when patients affected by this complication are those with pancreatic calcification, steatorrhea or endocrine insufficiency (9) due to long standing pancreatic disease. In such an instance, it would be logical to expect a deformed bile duct due to penetration by different degrees of fibrosis at different sites within the encasing pancreas, as seen in the pancreatic duct. Indeed, a minority of the common bile duct strictures in chronic pancreatitis suggest such a pathologic evolvement with their hourglass appearance (9,12). However, smooth, gradual tapering of the distal common bile duct suggests some degree of mural response instead. Both clinical and experimental evidence is present to support such a hypothesis. The encasement theory does not explain restenoses of biliary anastomoses outside the pancreas (13), nor is it consistent with the inflammatory findings in the bile duct and gallbladder of affected patients (14).

It is well known that patients with chronic pancreatitis experience acute inflammatory exacerbations during the course of their disease. Induced edematous pancreatitis with a supramaximal dose of Cerulein is a well known model in the rat (15). Pancreatitis of this extent causes reversible homogeneous ultrastructural changes in the rat pancreas (16-18), is a controlled model, can be repeated and is rarely fatal (19). The rat bile duct serves to collect both the bile secretion of the liver, and the pancreatic juice secreted by the pancreas lobules surrounding the duct on its course to the duodenum (20). The experiment protocol allowed sufficient time for the pancreas to recover from the acute insult produced by Cerulein and counteract biochemical disturbances attributable to external compression by the swollen pancreas lobules. Although there is evidence that acute hemorrhagic pancreatitis may evolve into chronic pancreatitis with duct obstructing strictures after intraductal administration of bile salts (21), in this study we did not aim to produce chronic pancreatitis, but to construct a model to follow common bile duct response to a standard inflammatory insult of increasing persistance. A similar model was employed by Vaquero et al. (22), who gave Cyclosporin to some groups in addition to Cerulein to cause a response simulating chronic pancreatitis. The model we constructed has enabled the desired insult, as evidenced by the degree of pancreatic edema shifting from mild to significant and by the degree of the biochemical response as the intensity of Cerulein administration increased. Rats that received repeated Cerulein insults had higher pancreatic injury responses and bile flow disturbances than both control and single application groups, as reflected in Tables 3 and 4.

There was no significant difference in the parameters between control animals and B2 animals, or between Groups B1 and B2, indicating that the usual pancreatic response to a single application of Cerulein recovers within a week. However, response to the insult was sustained as the Cerulein applications were repeated. This is similar to a previous experience that reports delayed regeneration after repeated Cerulein administrations (23). The alkaline phosphatase and bilirubin levels showed parallel increases that suggested a cholestatic state, again as the insult was repeated. There was no difference between the choledochal cellular infiltration between the groups, although polymorphonuclear infiltration was more prominent (n.s.) in B3 and B4 rats with sustained Cerulein insult. Significant edema in the common bile duct wall was present also, in 57% of the animals in group B4. However, there was no increase in the connective tissue and reticulum fiber content of the common bile duct, and fibrinogen was negative immunohistochemically in all groups.

Elsasser et al. (24) showed that hydroxyproline content, acinar cell destruction, cellular infiltration and collagen deposition persisted during the first week of repeated Cerulein insults, but repetitive induction of pancreatitis did not result in pancreatic fibrosis. A similar conclusion can be made for the bile duct response to repeated Cerulein insults as well. Although it has been shown that bile duct epithelial cells are destroyed after acute interstitial pancreatitis with intraperitoneal or intravenous dibutyltin dichloride (25), the present study failed to detect the results of such destruction in a Cerulein model. Nevertheless, the tendency of the microscopic findings and the presence of moderate to strong immunohistochemical positivity for type IV collagen at the wall of the common bile duct in animals with a persisting insult, albeit statistically non-significant, may suggest circumstantial evidence for doubt that encasement by a fibrotic pancreas is not the only explanation for common bile duct strictures in chronic pancreatitis.

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