Enzyme load in pancreatic acinar cells is increased in the early stages of acute pancreatitis induced by duct obstruction in rats

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ABSTRACT

Trypsinogen and amylase content has been analysed by flow cytometry in individual pancreatic cells from rats with acute pancreatitis induced by pancreatic duct obstruction, from the earliest stages to 48 h after obstruction. Parallel morphological studies of the pancreas by electron microscopy and analysis of various parameters for the diagnosis of pancreatitis will allow research into the possible relationship between intracellular enzyme load and the severity of pancreatitis. Progressive increases in amylase activity in ascites and plasma, the volume of ascites, haematocrit, vacuolization, oedema and macrophage infiltration were observed between 1.5 h and 12 h after duct obstruction. A progressive increase in enzyme content was also observed in individual acinar cells at this stage. Interestingly, the larger increase was for trypsinogen, so that the trypsinogen/amylase ratio was significantly increased in all acinar cells by 12 h after duct obstruction. This represents a risk factor for the development of pancreatitis. Sections of pancreas taken from rats that had duct obstruction for 48 h showed massive dilatation and disorganization of the endoplasmic reticulum, focal apoptosis and necrosis. These severe alterations would affect enzyme synthesis, as reflected by the significant decrease in the intracellular enzyme load observed at this stage. However, not all acinar cells were affected equally by the damage induced by pancreatitis: R1 cells appeared to be more sensitive than R2 cells. In conclusion, intracellular accumulation of digestive enzymes occurs at early stages of pancreatitis, and this effect is proportionally greater for trypsinogen, a finding that could explain the degree of severity achieved in the course of pancreatitis.

INTRODUCTION

Acute pancreatitis is generally considered to be an autodigestive disease in which the premature activation of proteolytic zymogens plays a central role in disease development [1–4]. Various studies have indicated that the lysosomal hydrolase cathepsin B can activate trypsinogen, and the subsequent production of active trypsin can lead to catalysis of the activation of other enzyme zymogens [3,5,6]. The concept of ‘autodigestion’ is based on the biochemical determination of digestive enzymes in pancreas homogenates [4,7] and pancreatic secretion from animals with experimentally induced pancreatitis [8–10], and on morphological observations of pancreatic tissue from patients at late stages of the disease [11,12]. Recently, changes in the enzyme content of isolated zymogen granules have been reported in rats in which acute pancreatitis was induced by retrograde taurocholate infusion [13] and supramaximal doses of caerulein [14]. However, no studies have focused on measuring the amounts and types of enzyme stored within pancreatic acinar cells during the development of acute pancreatitis.

Key words: acinar cells, acute pancreatitis, digestive enzymes.
Abbreviation: CCK, cholecystokinin.
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Since the most common type of acute pancreatitis in humans originates from the bile lithiasis, we chose pancreatic duct obstruction as an experimental model to induce pancreatitis in rats. Our aim was to measure the amounts of amylase and trypsinogen in acinar cells from the earliest stages of pancreatitis in order to investigate whether a relationship can be established between the intracellular enzyme load and the severity of pancreatitis. To address this question, we have used flow cytometry for the measurement of enzyme content in individual acinar cells, and both electron microscopy and biochemical assays to determine the severity of pancreatitis.

### MATERIALS AND METHODS

#### Chemicals

The following chemicals were supplied by Sigma Chemical Co.: BSA, collagenase type VII, soybean trypsin inhibitor, amino acid admixture and rabbit anti-amylase antiserum. Fix & Perm cell permeabilization kits and goat anti-rabbit immunoglobulin antiserum labelled with phycoerythrin were purchased from Caltag. Rabbit anti-trypsinogen antiserum was obtained in our laboratory as previously described [15]; cross-reactivity tests with other pancreatic enzymes demonstrated high specificity. All other reagents were obtained from Merck.

#### Surgical procedure

Male Wistar rats weighing 250–300 g were used. After overnight fasting, the rats were anaesthetized with ether and acute pancreatitis was induced by obstruction of the bile/pancreatic duct. For this, a median laparotomy was performed and the choledocus was tied at the distal part, close to its exit to the duodenum. Afterwards, the abdomen was closed with 6% polyester fibre sutures and the rats were replaced in their cages and allowed free access to food and water. In sham-operated animals, the choledocus was dissected but not ligated.

#### Animal groups

Rats were divided randomly into different groups. The measurement of various parameters associated with acute pancreatitis and electron microscopy studies were carried out using control animals (n = 9) and rats that had experienced pancreatic obstruction for 1.5 h (n = 9), 3 h (n = 7), 6 h (n = 7), 12 h (n = 8) and 48 h (n = 8). In addition, different animals were used for flow cytometric analysis: controls (n = 16), rats that had experienced pancreatic obstruction for 1.5 h (n = 7), 12 h (n = 6) and 48 h (n = 6), and sham-operated rats, which were also analysed after 1.5 h (n = 5), 12 h (n = 5) and 48 h (n = 5). Animal deaths were recorded at the end of each experimental period. The study protocol was approved by the animal studies committee of Salamanca University.

#### Collection of samples

After an overnight fast, the rats were anaesthetized with sodium pentobarbital (3 mg/100 g body weight, intraperitoneally), laparotomy was performed and the transperitoneal exudate (ascites) was collected from the abdominal cavity. Blood samples were taken by cardiac puncture at the end of each experiment to determine plasma amylase activity and haematocrit. Finally, the entire pancreas was removed to determine the percentage of fluid, for electron microscopy analysis or for isolation of pancreatic cells.

#### Assays

Amylase activity was determined in plasma and ascites using the method of Noelting and Bernfeld [16]. To measure the haematocrit, heparinized blood was centrifuged in microhaematocrit tubes at 4000 g for 5 min. The amount of tissue fluid was calculated by drying the pancreas at 90 °C for 48 h; the wet/dry pancreatic weight ratio was expressed as the percentage of fluid.

#### Electron microscopy studies

Portions of pancreatic tissue were examined by electron microscopy. Samples were pre-fixed with 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde at 4 °C for 2 h, and then post-fixed with 2% OsO₄ in the same buffer, dehydrated in ethanol and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate/lead citrate and examined using a Zeiss electron microscope (EM 900). Multiple blinded sections were evaluated, and representative findings were used for photoreprints. In order to determine the number of zymogen granules per cell, at least 5000 granules were counted, and the value was obtained from the ratio between the number of zymogen granules and the number of nuclei.

#### Preparation of isolated cells

After an overnight fast, the rats used for flow cytometric analysis were anaesthetized with sodium pentobarbital (3 mg/100 g body weight, intraperitoneally). A median laparotomy was performed, the bile duct was ligated at its exit from the liver and the main pancreatic duct was cannulated at its exit into the duodenum for perfusion of 5 ml of a solution containing 25 mM Hepes, pH 7.4, collagenase (40 units/ml), 0.1 mg/ml soybean trypsin inhibitor, 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 14 mM D-glucose, 2 mM glutamine, 2% (w/v) BSA and 2% (w/v) amino acid mixture. The solution was gassed with 95% O₂/5% CO₂, and all subsequent incubations were performed with this gas phase. The pancreas was digested at 37 °C in a shaking water bath (200 cycles/min) for 20 min, being washed with fresh collagenase solution every 5 min. Following gentle pipetting through tips of decreasing diameter.
Table 1 Peritoneal fluid, amylase in ascites and plasma, haematocrit, tissue fluid and survival rate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>1.5 h</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal fluid (ml)</td>
<td>0.4 ± 0.15 (6)</td>
<td>2.43 ± 0.41 (7)**</td>
<td>2.62 ± 0.37 (5)**</td>
<td>3.26 ± 0.31 (5)**</td>
<td>10.8 ± 0.89 (5)**</td>
<td>1.54 ± 0.26 (5)**</td>
</tr>
<tr>
<td>Ascites amylase (units)</td>
<td>0.03 ± 0.01 (5)</td>
<td>0.39 ± 0.08 (7)**</td>
<td>1.98 ± 0.48 (5)**</td>
<td>2.92 ± 0.50 (6)**</td>
<td>13.52 ± 1.12 (5)**</td>
<td>0.35 ± 0.11 (6)*</td>
</tr>
<tr>
<td>Plasma amylase (units/ml)</td>
<td>5.83 ± 0.3 (6)</td>
<td>7.17 ± 0.55 (7)*</td>
<td>11.51 ± 0.97 (5)**</td>
<td>16.51 ± 1.42 (5)**</td>
<td>20.20 ± 2.11 (5)**</td>
<td>8.64 ± 0.67 (6)**</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43.73 ± 1.06 (6)</td>
<td>46.50 ± 1.01 (6)</td>
<td>48.3 ± 1.22 (5)*</td>
<td>51.83 ± 1.14 (6)**</td>
<td>55.17 ± 2.43 (6)**</td>
<td>42.91 ± 0.8 (6)</td>
</tr>
<tr>
<td>Tissue fluid (%)</td>
<td>69.58 ± 1.04 (5)</td>
<td>72.51 ± 0.95 (9)</td>
<td>77.72 ± 0.87 (6)**</td>
<td>78.42 ± 0.90 (6)**</td>
<td>81.94 ± 1.34 (8)**</td>
<td>70.13 ± 2.05 (6)</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>100 (17)</td>
<td>100 (16)</td>
<td>100 (6)</td>
<td>100 (7)</td>
<td>100 (14)</td>
<td>75 (12)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for the numbers of animals indicated in parentheses. Significance of differences compared with controls (Student’s t test): *P < 0.05; **P < 0.01; ***P < 0.001

Progressive increases in the volume of the peritoneal exudate, amylase activity in plasma and peritoneal fluid, haematocrit and percentage of fluid in the pancreas were observed in rats with pancreatic duct obstruction between 1.5 h and 12 h after ligation of the choledochus (Table 1). The haematocrit and the amount of fluid in the pancreas had returned to control values by 48 h after duct obstruction, but the volume of peritoneal fluid and the amylase levels in plasma and ascites were still significantly higher than in controls at 48 h. From 24 to 48 h after duct obstruction, there was a 25% decrease in the survival rate.

Statistical analysis

Results are expressed as means ± S.E.M. The unpaired Student’s t test was applied in order to establish whether differences between controls and rats with pancreatic obstruction were statistically significant. This statistical test was also used for the comparison between the two subsets of pancreatic acinar cells differentiated by flow cytometry. P values of < 0.05 were considered to be statistically significant.

RESULTS

Electron microscopy studies (Figure 1) revealed initial interstitial oedema, macrophage infiltration, dilatation of the rough endoplasmic reticulum and vacuolization in sections of pancreas from rats with pancreatic duct obstruction for 1.5 h (Figure 1B). These alterations had become much more severe by 12 h after obstruction, when disruption of the basolateral membrane could be observed (Figure 1C). Pancreases from rats with pancreatic duct obstruction maintained over 48 h (Figure 1D) also showed some apoptotic nuclei and focal necrosis. Significant (P < 0.001) hypergranulation was
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Figure 1  Electron microscopic sections of pancreas from controls (A) and rats with pancreatic duct obstruction for 1.5 h (B), 12 h (C) and 48 h (D)
Obstruction induces dilatation of the endoplasmic reticulum (arrow), vacuolization (arrowhead), macrophage (M) infiltration, oedema (star) and the formation of apoptotic nuclei (A). Magnification: (A) × 1950; (B)–(D) × 2860.

Figure 2  Numbers of zymogen granules (ZG) in individual acinar cells
Values are expressed as means ± S.E.M. Unpaired Student’s t tests revealed statistically significant differences compared with controls (** * * * P < 0.001).

observed after 12 h of pancreatic duct obstruction, but the number of zymogen granules had decreased significantly (P < 0.001) by 48 h after ligation (Figure 2).

Figure 3  Flow cytometric distribution of pancreatic cells from control rats
FSC, forward scatter; SSC, side scatter. R1 and R2 represent distinct cell populations. The dot plot is a representative example from one experiment.
Intracellular enzymes in acute pancreatitis

Figure 4 Flow cytometric analysis of trypsinogen and amylase content in individual acinar cells from sham-operated rats and duct-obstructed rats at 1.5 h, 12 h and 48 h

Solid bars, sham-operated rats (n = 5 in each group); patterned bars, duct-obstructed rats (1.5 h, n = 7; 12 and 48 h, n = 6). Values are means ± S.E.M. Unpaired Student’s t tests revealed statistically significant differences compared with sham-operated rats (**P < 0.01; ***P < 0.001).

Figure 3 shows a representative example of the flow cytometric distribution of pancreatic cells on the basis of light scatter. Two well-differentiated populations could be observed in control, sham-operated and pancreatic-duct-ligated-rats at 1.5 h, 12 h and 48 h after ligation, as defined by forward or low-angle light scatter and side or 90° light scatter, which are referred to as R1 and R2.

Flow cytometric analysis of individual cells after staining with specific antisera against trypsinogen and amylase revealed a progressive increase in the intracellular enzyme content over the 12 h after duct obstruction (Figure 4). A significant rise in trypsinogen content was found at 1.5 h and 12 h after duct obstruction in both R1 and R2 cells, but the increase in amylase only reached statistical significance in R2 cells. The contents of both amylase and trypsinogen were significantly reduced in all acinar cells by 48 h after pancreatic obstruction.

With regard to enzyme labelling, both R1 and R2 cells displayed a high degree of heterogeneity; however, in both controls and sham-operated rats, R1 cells showed similar mean levels of amylase and trypsinogen to R2 cells (Table 2). In contrast, a significantly higher enzyme content was found in R2 cells compared with R1 cells in rats with duct obstruction, at all stages.

Analysis of the trypsinogen/amylase ratio (Figure 5) revealed a significant increase (P < 0.001) in both R1 and R2 cells from rats with pancreatic obstruction maintained for 12 h.

DISCUSSION

The model of pancreatitis in rats induced by pancreatic duct obstruction [18,19] represents a useful experimental corollary of gallstone-induced acute pancreatitis in humans with which to investigate disease pathogenesis. The results obtained in the present study may help towards our understanding of the events leading to the

Table 2 Trypsinogen and amylase content in R1 and R2 cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Controls (n = 12)</th>
<th>Sham-operated</th>
<th>Duct obstruction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 h (n = 5)</td>
<td>12 h (n = 5)</td>
<td>48 h (n = 5)</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1 cells</td>
<td>1.97 ± 0.03</td>
<td>1.90 ± 0.07</td>
<td>1.99 ± 0.09</td>
</tr>
<tr>
<td>R2 cells</td>
<td>1.96 ± 0.02</td>
<td>1.92 ± 0.06</td>
<td>1.95 ± 0.1</td>
</tr>
</tbody>
</table>

| Amylase |
| R1 cells   | 7.72 ± 0.10      | 7.98 ± 0.12   | 7.99 ± 0.23     | 8.20 ± 0.20   |
| R2 cells   | 8.20 ± 0.25      | 7.85 ± 0.10   | 8.13 ± 0.20     | 7.96 ± 0.24   |

Values are expressed as 10⁻² × no. of cells in the fluorescence channel, and are means ± S.E.M. (n = number of experiments). Unpaired Student’s t tests revealed statistically significant differences between R1 and R2 cells: *P < 0.05; **P < 0.01; ***P < 0.001.

10⁻² × Cell no.

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development of pancreatitis, since changes in the intracellular enzyme content could be detected from a very early point after duct obstruction through to the stage at which pancreatitis is clearly established. Usually it is impossible to obtain clinical material during the early stages of pancreatitis, which is precisely the most interesting period, in which the severity of disease may be determined [13,20].

Various morphological alterations were observed from the early stages after pancreatic duct obstruction, such as dilatation of the rough endoplasmic reticulum, vacuolization and inflammation. Previous studies [7,21–23] have suggested that the high pressure developed in the choledocus may be an important factor that leads to the development of these alterations. However, such changes have also been described in other experimental models of pancreatitis [13,24,25], and so other factors should also be taken into account.

Flow cytometry analysis allowed us to differentiate two types of cell according to their properties of light scatter, which are grouped in two populations. In control rats, the two types of cells had the same enzyme content, a result that supports the notion that all acinar cells potentially are equally efficient in secreting any digestive enzyme. These observations are not consistent with the hypothesis of Gingras and Bendayan [26], who suggested the existence of acinar cells containing different ratios of enzymes, and that the preferential activity of some cells could explain non-parallel secretion in response to specific stimuli [15,27]. Our results support an alternative hypothesis, whereby non-parallel secretion is due to the selective exocytosis of heterogeneous zymogen granules with different enzyme ratios [15,28], which would be shared by all acinar cells. Results from biochemical assays of pancreatic trypsinogen and amylase levels have previously validated the flow cytometry data [15].

Flow cytometry analysis revealed an increase in the quantities of enzymes in individual cells from rats with acute pancreatitis from the earliest stages to 12 h after duct obstruction. The accumulation of intracellular enzymes could be explained by two possibilities: (1) the blockage of pancreatic secretion produced by acute pancreatitis, which has been observed both clinically [29] and experimentally [8,30–33], and (2) an increase in plasma levels of cholecystokinin (CCK) as a consequence of the absence of the feedback inhibitory mechanism induced by bile [18] and pancreatic proteases [34] in the duodenum. Previous studies have reported that CCK plasma levels are increased in rats from 1 h [35] to at least 18 h [36] after pancreatic duct obstruction. Interestingly, a greater increase in trypsinogen content than in amylase content was observed in individual cells, which could be explained by the preferential stimulatory effect of CCK on the synthesis of proteases [15,37,38]. This enzyme imbalance in each cell will represent a high risk, because the intracellular activation of such a high trypsinogen load would lead to the rapid autodigestion of the gland, a process that could be carried out in the great vacuoles observed in pancreatic sections from rats with pancreatic obstruction [9,18,39]. The large amount of secretory products that accumulate within acinar cells would result in the release of some of them into the interstitial space. As a consequence of this, an increase in amylase activity in the plasma and the peritoneal exudate was observed, which reached a maximum 12 h after duct obstruction. Severe pancreatic oedema and a high volume of fluid in the peritoneal cavity were also observed at this stage, which in turn will induce hypovolaemia associated with significant increases in the haematocrit.

Hypogranulation and a decrease in the intracellular enzyme content were observed in rats with pancreatic obstruction maintained over 48 h. These findings could not be explained by the release of enzymes into the interstitium, but rather by ineffective enzyme synthesis as a consequence of the severe alterations induced by acute pancreatitis, such as massive dilatation of the rough endoplasmic reticulum and the anomalous distribution of chromatin in the nuclei. Apoptosis [21,40,41] and focal necrosis [19,35,36], described previously in rats with pancreatitis induced by obstruction, were also observed in the present study 48 h after duct obstruction. Failings in mechanisms of enzyme synthesis related to amino acid uptake have been reported previously in different experimental models of pancreatitis [42,43]. Modifications at the transcriptional and post-transcriptional levels that lead to changes in the normal pattern of pancreatic gene expression were also reported by Iovanna et al. [44], who described a decrease in digestive enzyme content of over 50 % in rats 48 h after induction of acute pancreatitis by retrograde infusion of taurocholate.

Interestingly, in contrast with the observations made in control and sham-operated animals, not all acinar cells from rats with obstruction-induced pancreatitis at any stage showed the same intracellular enzyme content. This finding suggests that the balance between the synthesis and discharge of enzymes is not uniform in the whole pancreas, perhaps due to the fact that the different pancreatic cells are not equally affected by pancreatitis; thus R1 cells appeared to be more sensitive to the damage produced by duct obstruction.

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