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Fragility of Subcellular Organelles Induced by Pancreatic Duct Obstruction in Rabbits

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Summary

The effects of short-term (6 hours) pancreatic duct obstruction on the possible secretion of lysosomal enzyme into pancreatic juice and subcellular lysosomal and mitochondrial fragility were investigated in rabbits. Caerulein stimulated the secretion of amylase and cathepsin B into pancreatic juice in controls. Blockage of the pancreatic duct for 6 hours caused a significant decrease of amylase and cathepsin B output into pancreatic juice, and a significant rise in portal serum amylase and cathepsin B levels, and pancreatic amylase content and an accelerated leakage of cathepsin B from lysosomes and malate dehydrogenase from mitochondria in in-vitro preparations, as well as redistribution of cathepsin B in acinar cells. These changes tended to continue for up to 12 hours after removal of the pancreatic duct obstruction. These results indicate that under physiological conditions, lysosomal enzymes are secreted into the pancreatic juice in response to stimulation by gut hormones and that increased lysosomal fragility, mitochondrial fragility and impaired pancreatic energy charge levels are closely related to the pathogenesis of pancreatic injury in this model. Moreover, zymogen colocalized with lysosomal enzyme after duct obstruction was secreted into pancreatic juice in increased amount together with digestive enzymes; these findings suggest that lysosomal enzymes play important pathophysiological roles in pancreatic juice.

Introduction

Both morphological and biochemical studies have shown that, in the early stage, two forms of experimental pancreatitis (diet-induced[16, 23], and secretagogue-induced[28, 29, 30, 35] share the common attribute of co-localization of digestive enzymes with lysosomal hydrolases inside large cytoplasmic vacuoles[32], and the subcellular fractionation of acinar cells has demonstrated redistribution of lysosomal enzymes from the lysosome-rich to the zymogen granule-rich fraction. Since cathepsin B, a lysosomal enzyme can activate trypsinogen[12, 14, 26], and trypsin can activate the other pancreatic digestive enzymes, the co-localization of digestive enzymes with lysosomal hydrolases could lead to the activation of intra-cellular digestive enzymes and cause increased lysosomal fragility as well as increased fragility of other subcellular organelles, such as mitochondria, and impaired pancreatic

Key words: Pancreatic duct obstruction, Lysosome, Mitochondria, Cathepsin B, Malate dehydrogenase.
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energy metabolism and might be an important trigger in the development of acute pancreatitis inside the acinar cells. Thus lysosomal enzymes seem to play an important role in the pathogenesis of acute pancreatitis[33].

On the other hand, gallstone pancreatitis, which is the most common form of acute pancreatitis in humans, seems to be triggered by the passage through or incarceration of a stone in the terminal bile duct[1, 3, 10, 13, 22, 24]. However, the mechanism whereby pancreatic duct obstruction induces pancreatitis has not been clarified.

In this study, we examined the secretion of lysosomal enzymes into pancreatic juice during stimulation with pancreatic secretagogues shortly after obstruction of the pancreatic duct, as well as lysosomal fragility, mitochondrial fragility, and pancreatic energy metabolism. The time course changes in these parameters in the stage of recovery from the pancreatic damage were also investigated.

Materials and Methods

Animal preparation

Forty-four New Zealand white rabbits weighing 2.45 to 2.80 kg (Shizuoka Experimental Animals, Shizuoka, Japan) were used in this study. They were kept in light-dark cycle regulated (light: 5:00–17:00) and air-conditioned (23±3°C) animal quarters in our university before the experiments and allowed to become aclimatized to standard laboratory conditions for at least 4 days. The rabbits were maintained throughout the study in accordance with the guidelines of the Committee on Animal Care of Kyoto University, Kyoto, Japan. Tests were performed after a 16-hour fast, starting at 8:00–9:00 AM to rule out the effects of circadian rhythm on the exocrine pancreas. Anesthesia was induced by the intravenous administration of sodium pentobarbital (35 mg/kg) through an ear vein and maintained by periodic intravenous injections of pentobarbital (10 mg/kg). Animals were kept on heating pads at 40°C (American Medical Systems, Cincinnati, OH, U.S.A.) and under overhead lamps to maintain their body temperature. Before opening of the abdomen, a catheter (V-3 catheter, Insul-Tab, Woburn, MA, U.S.A.) was passed through the right femoral vein into the inferior vena cava. A midline skin incision was made from the xiphoid process to the umbilicus, another catheter (Medicut 18-gauge catheter, Sherwood Medical Industries, St. Louis, MO, U.S.A.) was placed in the portal trunk via a branch of the ileocaecal vein for portal blood samplings. At this moment, all the animals were divided into two groups: pancreatic duct obstruction (PDO) group, and control group (CONT). All the animals were infused with heparinized (30 IU/ml) saline at a rate of 1.58 ml/hr during the operation.

In 24 PDO rabbits, the pancreatic duct was ligated just adjacent to the duodenum with a metal clip for 6 hours. After ligation of the pancreatic duct, the abdomen was closed and anesthesia was maintained. After 6 hours, portal blood (1.0 ml) was drawn for the estimation of pancreatic digestive enzyme and lysosomal enzyme levels, and the metal clip on the pancreatic duct was removed. Six rabbits were used for each of the following experiments: 0, 12, 24, and 36 hours after release of the pancreatic duct ligation. After removal of the metal clip, abdomen was closed, and were given nothing p.o, and an infusion of heparinized (30 IU/ml) lactate-Ringer solution containing 10% glucose was continued at a speed of 1.58 ml/hr by infusion pump (Harvard Apparatus, South Natick, MA, U.S.A.), and the portal catheter was locked by heparinized saline (100 IU/ml). In the 20 control rabbits, the pancreatic duct near the duodenum was manipulated gently with cotton swabs

and the abdomen was closed; anesthesia was maintained for 6 hours. Then, the abdomen was reopened, and the pancreatic duct near the duodenum was again manipulated gently. Five rabbits were used for each of the following experiments: 12 hours, 24 hours, and 36 hours after the second manipulation. After operation, these control animals were given nothing p.o.

Portal serum amylase and cathepsin B levels

Before ligation of the pancreatic duct, 6 hours after ligation, and 12, 24, and 36 hours after removal of the ligation, portal blood was drown, and serum amylase and cathepsin B, as a lysosomal enzyme, levels were measured.

In-vivo secretion of amylase and cathepsin B into pancreatic juice

For each group, at each stage after 6 hours of pancreatic duct obstruction, and after removal of the pancreatic duct ligation, the abdomen was reopened, the pylorus was ligated, and a cahteter (PE 70, Clay Adams, Parsippany, NJ, U.S.A.) was inserted through a gastrostomy to draw off gastric juice. Another catheter (PE 50) was inserted into the pancreatic duct just adjacent to its opening into the duodenum for a distance of 5 mm for the collecting of pancreatic juice. During this experiment, anesthesia was maintained and heparinized saline was infused at a speed of 1.58 ml/hr. The external portion of the catheter in the pancreatic duct was placed on a sponge pad in a natural position, and one portion of the abdominal wound was left open to check the position of the catheter. The rest of the abdominal wound was closed and covered with gauze soaked in warm saline. In addition, the animals were kept warm by heating pads and overhead lights during the experiment. After a 30 min period of stabilization, all the animals were infused with secretin (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of 0.2 CU/kg・hr for 2 hours, and with caerulein (Sigma Chemical Co.) for the next 2 hours at a dose of 0.2 μg/kg・hr. Pancreatic juice was collected hourly (fractions, S_1, S_2, C_1 and C_2) in preweighed Eppendorf tubes on ice, and the volume of pancreatic juice was determined by direct weighing on an automatic electric balance, by substracting the tube weight and adjusting the density of the juice to 1.0 g/ml. Amylase and cathepsin B activity in each fraction was measured and the output of both enzymes was expressed as U/kg・hr. Immediately after collection, each fraction was placed in the refrigerator (0°C), and cathepsin B activity was measured on the day of the experiment. All the animals used in this experiment were killed by a large dose of pentobarbital and blood withdrawed from the inferior vena cava and prepared for the next experiment.

Pancreatic water content

After removal of the pancreas, one small part was used for quantitation of the pancreatic water content by a comparison of the weight immediately after removal (wet weight) with the weight after desiccation at 150°C for 48 hours in a desiccator (Isotemp® Oven, Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Histological examination

Other small portions of pancreas were fixed overnight by immersion in phosphate-buffered 10% neutral formalin. After paraffin embedding, sectioning and staining with hematoxylin-eosin, the sections were examined light microscopically by a blinded observer who graded acinar cell changes such as interstitial edema, acinar cell vacuolization and inflammatory cell infiltration.

Pancreatic amylase and cathepsin B content

About one fourth of the pancreas was used for the determination of pancreatic content of amylase and cathepsin B. This portion was homogenized in cold phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100 (Fisher Scientific) in a Polytron homogenizer (Brinkmann Instrument, Westbury, NY, U.S.A.), and unbroken cells and debris were removed by low speed centrifuga-

tion (150 × g, 15 min, 4°C). Amylase activity, cathepsin B activity, and deoxyribonucleic acid (DNA) concentration were measured in the resulting supernatant. The pancreatic amylase and cathepsin B contents were expressed as U/mg · DNA.

Subcellular distribution of cathepsin B

Another one fourth of the gland was used for subcellular fractionation and determination of the distribution of cathepsin B in acinar cells. The excised, trimmed and homogenized rabbit pancreas was separated into various subcellular fractions by differential centrifugation. The protocol originally developed by TARTAKOFF and JAMIESON[34] with modifications for studies of rat tissue[8], was further modified to permit optimum secaration of rabbit pancreatic cell fractions. Briefly, pancreatic fragments were homogenized in 6 ml of cold 0.3 M sucrose solution with three up-and-down storkes of a Dounce homogenizer (Wheaton, Milleville, NJ, U.S.A.). The resulting homogenate was centrifuged (150 × g, 10 min, 4°C) to pellet debris and unbroken cells, which were discarded. The supernatant after this low speed centrifugation was considered to contain 100% of each of the components measured. This supernatant was centrifuged (1000 × g, 15 min, 4°C) to obtain a zymogen granule-rich pellet (1.0 KP), and the supernatant was centrifuged again (12000 × g, 12 min, 4°C) to yield a lysosome-and mitochondria-rich pellet (12 KP) and a supernatant, which was considered to contain the microsomal and soluble fraction (12 KS). The various pellets obtained during fractionation were resuspended individually in 2 ml of cold (4°C) 0.3 M sucrose solution, and cathepsin B activity in each fraction was measured and expressed as a percentage of the total activity.

In-vitro incubation of pancreatic lysosome and mitochondria

Another one fourth of the pancreas was used for in-vitro pancreatic lysosome and mitochondria incubation experiments. The pancreatic tissue was homogenized in 6 ml of ice-chilled 0.3 M sucrose solution with a Dounce homogenizer, and the lysosome-and mitochondria-rich fraction was obtained as described in the forementioned protocol. This lysosome-and mitochondria-rich fraction, arbitrarily considered to contain 100% of both lysosomal and mitochondrial enzyme activities, was resuspended in the same 0.3 M sucrose buffer and incubated for varying intervals (30, 60 and 90 min) at 25°C in a shaking water bath in room air. The samples were then recentrifuged (12,000 × g, 12 min, 4°C) to separate the particulate from the soluble lysosomal and mitochondrial enzyme activities, each of which was measured individually after separation of the pellet and supernatant. As a lysosomal enzyme, cathepsin B activity was measured in both the pellet and soluble fractions.

Centrifugation and subsequent measurement of particulate and soluble lysosomal enzyme activities identified the rate and extent of in-vitro rupture of lysosomal enzyme-containing organelles. Soluble cathepsin B activity, expressed as a percentage of total cathepsin B activity, was used as an index of lysosomal fragility. In the same samples, malate dehydrogenase (MDH) activity as a mitochondrial enzyme was measured and the MDH leakage from mitochondria was expressed in the same way as cathepsin B leakage as an index of mitochondrial fragility.

Pancreatic adenylate energy charge levels

The remaining one fourth of the pancreas was used in this experiment. The pancreas tissue was frozen in liquid nitrogen, then powdered with a mortar and pestle in a liquid nigrogen bath and homogenized in 4 times their volume of chilled 6% (W/V) perchloric acid containing 1 mM edetic acid. The extract was centrifuged at 10000 × g at 0°C for 15 min. The supernatant was adjusted to pH 6.0 with cold 69% (W/V) K_2CO_3 and recentrifuged at 10000 × g at 0°C for 5 min. The final supernatant was used to determine the concentration of adenine nucleotides (ATP, ADP, and AMP) in pancreatic tissue. The energy charge was calculated by the formula proposed by ATKINSON[4]:

energy charge=(ATP+0.5 ADP)/(ATP+ADP+AMP).

Assays

Amylase activity was measured with soluble starch (Sigma Chemical) as the substrate by the method of BERNFELD[6], and one unit (U) of activity was defined as that which can liberate 1 mg of maltose from the substrate per min at 30°C. Cathepsin B activity was measured fluorometrically by the method of McDONALD and ELLIS[21] with CBZ-2-arginyl-arginine-B-naphthylamide (Bachem Bioscience, Philadelphia, PA, U.S.A.) as the substrate, and one unit (U) of cathepsin B activity was defined as that which releases 1 nanomole of β-naphthylamine (Sigma Chemical) per min from the substrate at 37°C. Deoxyribonucleic acid (DNA) concentration was measured fluorometrically by the method of LABARCA and PAIGEN[19] with cald thymus DNA (Sigma Chemical) as the substrate. Malate dehydrogenase (MDH) was measured spectrometrically by the method of BERGMEYER (5). ATP was measured enzymatically by the method of LAMPRECHT and TRAUTSCHOLD[20], and ADP and AMP by that of JAWOREK and co-workers[15].

Data presentation

The results reported in this communication represent the mean±SEM for n determinations. Differences between groups were evaluated by analysis of variance, and significant differences were defined as those associated with probability value (P) of less than 0.05.

Results

Portal blood serum amylase and cathepsin B levels

Six-hour pancreatic duct obstruction caused very significant portal hyperamylasemia (54±7 U/ml) (before obstruction, 13±2 U/ml; control group 15±2 U/ml), but 12 hours after release of the pancreatic duct obstruction (PDO), portal blood amylase levels (16±3 U/ml) tended to return to the control values (14±2 U/ml). At 24 hours and 36 hours after release of the PDO, there were no significant differences between the PDO group (24 hours, 14±2 U/ml; 36 hours, 10±2 U/ml) and the control gorup (24 hours, 11±2 U/ml; 36 hours, 13±2 U/ml) (Fig. 1a). Portal serum cathepsin B levels showed the same changes as amylase levels, and 6-hour PDO caused a marked and significant increase in portal serum cathepsin B levels, (8.4±0.8 U/ml; before PDO, 1.8±0.3 U/ml; control group, 2.4±0.3 U/ml). But by 12 hours after the release of the PDO, this high level of cathepsin B in the portal blood had almost disappeared (2.6±0.6 U/ml; control group 2.0±0.2 U/ml). There were no significant differences between the PDO group (24 hours, 2.2±0.5 U/ml; 36 hours, 2.0±0.4 U/ml) and the control group (24 hours, 2.3±0.4 U/ml, 36 hours, 1.9±0.2 U/ml) 24 hours and 36 hours after the release of the PDO (Fig. 1b).

In-vivo secretion of amylase and cathepsin B into pancreatic juice

After pancreatic duct obstruction (PDO) for 6 hours, the volumes of pancreatic juice in both the secretin and caerulein fractions were significantly lower than in the control group, and these changes tended to continue for 12 hours after release of the PDO, with no significant differences 12 hours later. Although there were no significant differences between the PDO group and the control group, the volume in the PDO group tended to be slightly more than in the control group 24 hours after release of the PDO. At 36 hours after the release of the PDO, there were no significant differences between the two groups (Table I). A 6-hour PDO caused a remarked and significant decrease in amylase output into pancreatic juice in both the secretin and caerulein fractions (S_1, 27±8 U/kg・hr; S_2, 31±7 U/kg・hr; C_1, 343±27 U/kg・hr; C_2, 426±35 U/kg・hr, control S; S_1, 65±12 U/kg

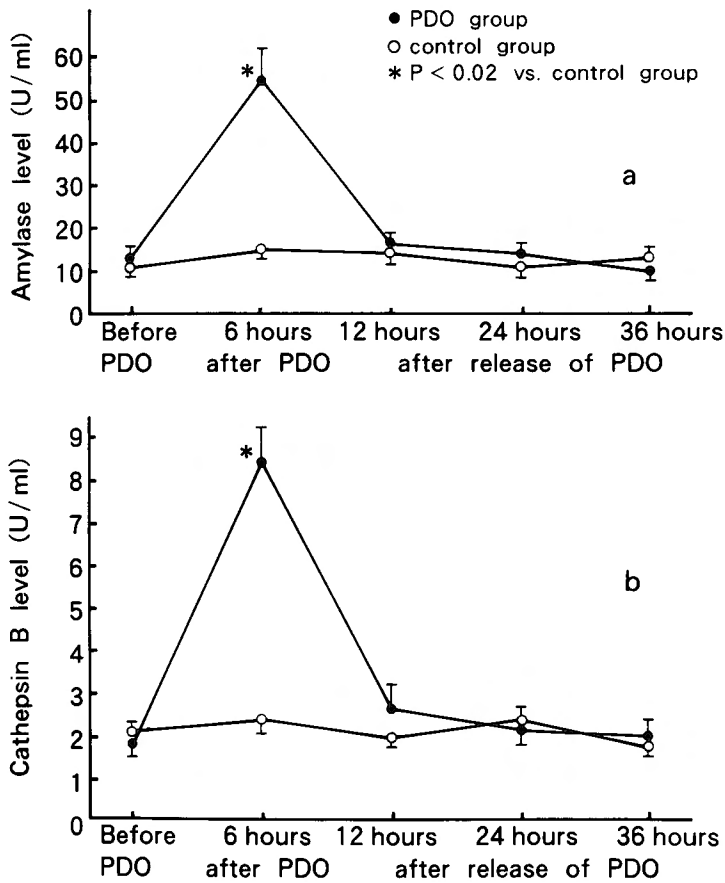

Fig. 1 Effects of pancreatic duct obstruction (PDO) on changes in portal amylase levels (a) and portal cathepsin B levels (b) in rabbits
There were 6 animals at each stage in the PDO group, and 5 animals at each stage in the control group. mean±SEM.

Table 1 Effects of pancreatic duct obstruction on changes in pancreatic juice volume.

Stage of experiment	Group	n	Pancreatic juice volume (ml/kg · hr) Pancreatic juice fractions			
			S_1	S_2	C_1	C_2
6 hours after pancreatic duct obstruction	PDO	6	0.58±0.05*	0.63±0.08*	0.72±0.07*	0.69±0.05*
	CONT	5	1.07±0.06	1.04±0.09	1.13±0.08	1.18±0.07
12 hours after removal of pancreatic duct obstruction	PDO	6	0.94±0.07	0.99±0.06	0.98±0.07	0.91±0.09
	CONT	5	1.12±0.08	1.07±0.05	1.14±0.09	1.09±0.07
24 hours after removal of pancreatic duct obstruction	PDO	6	1.20±0.09	1.17±0.06	1.24±0.12	1.21±0.10
	CONT	5	1.05±0.04	1.08±0.07	1.16±0.08	1.18±0.05
36 hours after removal of pancreatic duct obstruction	PDO	6	1.12±0.06	1.15±0.08	1.19±0.10	1.13±0.06
	CONT	5	1.06±0.09	1.10±0.05	1.22±0.09	1.17±0.08

The volume of pancreatic juice is expressed as ml/kg · hr in each fraction. (S_1 and S_2, secretin fractions at 1 and 2 hours, C_1 and C_2, caerulein fractions at 1 and 2 hours). PDO, pancreatic duct obstructed group; CONT, control group; *, $p<0.02$ compared with CONT group.

・hr, S_2, 73±15 U/kg・hr; C_1, 627±28 U/kg・hr; C_2, 654±31 U/kg・hr). At 12 hours after the release of the PDO, this impaired amylase output tended to recover (S_1, 43±15 U/kg・hr; S_2, 53±19 U/kg・hr; C_1, 492±38 U/kg・hr; C_2, 504±25 U/kg・hr; control S; S_1, 58±13 U/kg・hr, S_2, 64±18 U/kg・hr; C_1, 543±24 U/kg・hr; C_2, 582±34 U/kg・hr). On the contrary, 24 hours after the release of the PDO, amylase output in the PDO group was significantly increased in both the secretin and caerulein fractions (S_1, 124±22 U/kg・hr; S_2, 128±19 U/kg・hr; C_1, 984±41 U/kg・hr; C_2, 928±46 U/kg・hr; control S: S_1, 75±13 U/kg・hr; S_2, 66±20 U/kg・hr; C_1, 576±26 U/kg・hr; C_2, 551±28 U/kg・hr), but 36 hours after the release of the PDO, there were no significant differences between the PDO group (S_1, 75±21 U/kg・hr; S_2, 68±18 U/kg・hr; C_1, 624±48 U/kg・hr; C_2, 643±28 U/kg・hr) and the control group (S_1, 68±13 U/kg・hr; S_2, 61±15 U/kg・hr; C_1, 597±38 U/kg・hr; C_2, 565±31 U/kg・hr) (Fig. 2a, b, c, d).

Cathepsin B output into pancreatic juice showed the same changes, with only a little activity in the secretin fractions, but considerable activity in the caerulein fractions. PDO for 6 hours caused a significant decrease in cathepsin B output in both the secretin fraction (S_1, 0.4±0.1 U/kg・hr; S_2, 0.3±0.1 U/kg・hr) and the caerulein fraction (C_1, 8.2±0.7 U/kg・hr; C_2, 8.9±1.1 U/kg・hr) compared with the control group (S_1, 0.9±0.2 U/kg・hr; S_2, 0.8±0.4 U/kg・hr; C_1, 14.3±1.8 U/kg・hr; C_2, 13.7±1.4 U/kg・hr). By 12 hours after the release of the PDO, the reduced output of cathepsin B had recovered somewhat (S_1, 0.5±0.2 U/kg・hr; S_2, 0.6±0.2 U/kg・hr; C_1, 10.6±1.8 U/kg・hr; C_2, 11.5±2.2 U/kg・hr; control S: S_1, 0.8±0.2 U/kg・hr; S_2, 1.0±0.2 U/kg・hr; C_1, 12.8±1.2 U/kg・hr; C_2, 13.1±2.1 U/kg・hr). However, by 24 hours after the release of the PDO, cathepsin B output in the PDO group was significantly higher (S_1, 2.4±0.4 U/kg・hr; S_2, 3.1±0.5 U/kg・hr; C_1, 21.4±2.1 U/kg・hr; C_2, 22.5±2.7 U/kg・hr) than in the control group (S_1, 0.7±0.3 U/kg・hr; S_2, 0.8±0.2 U/kg・hr; C_1, 14.0±1.4 U/kg・hr; C_2, 13.9±2.3 U/kg・hr) and by 36 hours after the release of the PDO, there were no significant differences between the two

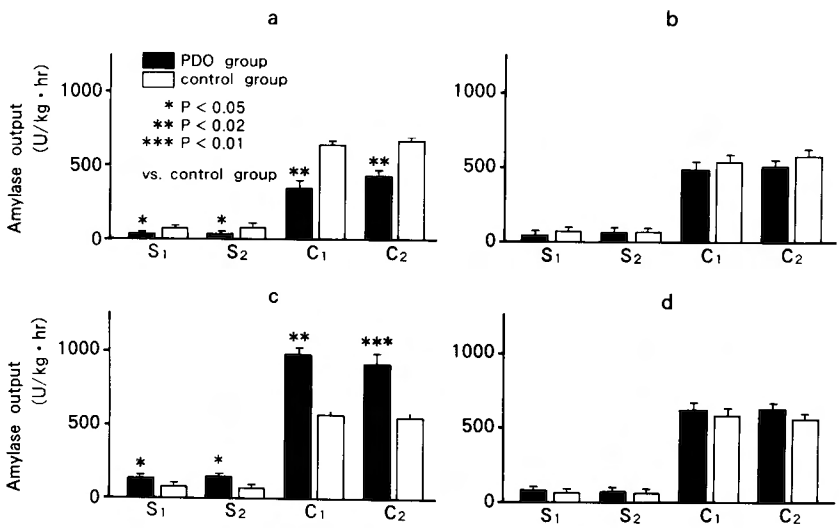

Fig. 2 Effect of PDO on changes in amylase output stimulated by secretin (0.2 CU/kg・hr) and caerulein (0.2 μg/kg・hr) in rabbits; (a) 6 hours after PDO, (b) 12 hours after release of PDO, (c) 24 hours after release of PDO, (d) 36 hours after release of PDO
There were 6 rabbits in the PDO group and 5 rabbits in the control group at each stage. mean±SEM.

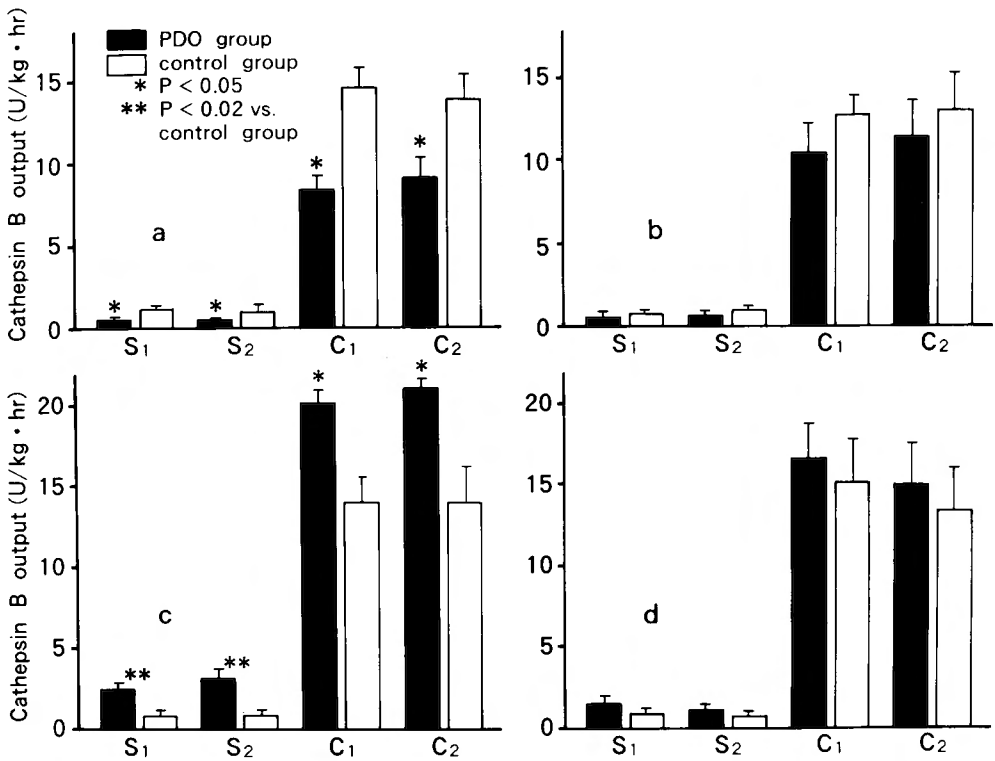

Fig. 3 Effect of PDO in cathepsin B output stimulated by secretin (0.2 CU/kg · hr) and caerulein (0.2 μg/kg · hr) in rabbits; (a) 6 hours after PDO, (b) 12 hours after release of PDO, (c) 24 hours after release of PDO, and (d) 36 hours after release of PDO
There were 6 rabbits in the PDO group, and 5 rabbits in the control group at each stage.

Table 2 Effects of pancreatic duct obstruction on changes in pancreatic water content.

Stage	Group	n	Pancreatic water content (%)
6 hours after pancreatic duct obstruction (PDO)	PDO	6	84±2*
	CONT	5	76±2
12 hours after removal of PDO	PDO	6	81±2
	CONT	5	77±2
24 hours after removal of PDO	PDO	6	78±2
	CONT	5	74±1
36 hours after removal of PDO	PDO	6	79±2
	CONT	5	75±1

The pancreatic water content is expressed as a % of total wet weight. PDO, pancreatic duct obstructed animals; CONT, control laparotomy group; *, $p<0.05$ compared with CONT group.

Table 3 Effects of pancreatic duct obstruction on changes in pancreatic histology.

Histological findings		Stage			
		6 hours after pancreatic duct obstruction (PDO)	12 hours after removal of PDO	24 hours after removal of PDO	36 hours after removal of PDO
Interstitial edema	PDO 6	1.5±0.2**(1–2)	0.7±0.2*(0–1)	0.3±0.2 (0–1)	0 (0)
	CONT 5	0 (0)	0 (0)	0 (0)	0 (0)
Acinar cell vacuolization	PDO 6	0.7±0.2 (0–1)	0.2±0.2 (0–1)	0 (0)	0 (0)
	CONT 5	0 (0)	0 (0)	0 (0)	0 (0)
Inflammatory cell infiltration	PDO 6	0.3±0.2 (0–1)	0 (0)	0 (0)	0 (0)
	CONT 5	0 (0)	0 (0)	0 (0)	0 (0)

Histologic changes were graded blindly on a score from 0 (no change) to 4+ (maximum change), and the values were expressed as the mean±SEM of scores, ( ), range of scores; PDO, pancreatic duct obstructed animals; CONT, control laparotomy animals; *, $p<0.02$; **, $p<0.01$.

Table 4 Effect of pancreatic duct obstruction on changes in pancreatic amyalse and cathepsin B content.

Pancreatic content	Group n	Stage			
		6 hours after pancreatic duct obstruction (PDO)	12 hours after removal of PDO	24 hours after removal of PDO	36 hours after removal of PDO
Pancreatic amylase content (U/mg DNA)	PDO 6	624±48**	504±38*	446±33	421±28
	CONT 5	396±29	418±32	407±26	384±25
Pancreatic cathepsin B content (U/mg・DNA)	PDO 6	1526±204	1324±189	1218±147	1193±154
	CONT 5	1189±175	1245±131	1221±163	1172±158

Amylase and cathepsin B content is expressed as U/mg・DNA. *, $p<0.05$; **, $p<0.02$ compared with CONT group.

groups (PDO: S_1, 1.5±0.4 U/kg・hr; S_2, 1.1±0.3 U/kg・hr; C_1, 16.6±2.2 U/kg・hr; C_2, 15.1±2.4 U/kg・hr; control S: S_1, 0.9±0.2 U/kg・hr; S_2, 0.7±0.2 U/kg・hr; C_1, 15.2±2.6 U/kg・hr, C_2, 13.5±2.7 U/kg・hr) (Fig. 3a, b, c, d).

Pancreatic water content

Pancreatic duct obstruction (PDO) for 6 hours caused some increase in the pancreatic water content (84±2%, significantly higher than in the control group, 76±2%. This increased pancreatic water content was not obvious macroscopically. By 12 hours after the release of the PDO, this increased pancreatic water content had almost disappeared and at 24 hours and 36 hours, there was no significant difference between the two groups (Table II).

Histological examination

Histological examination after pancreatic duct obstruction (PDO) for 6 hours showed mile interstitial edema and slight acinar cell vacuolization-significant changes compared with the control group. Inflammatory cell infiltration was not significantly different at any stage. By 12 hours after the release of the PDO, there was still slight, but significant, interstitial edema, but acinar cell vacuolization had almost disappeared. At 24 hours and 36 hours, there were no significant differences in interstitial edema or acinar cell vacuolization between the two groups (Table III).

Pancreatic amylase and cathepsin B content

Pancreatic duct obstruction (PDO) for 6 hours caused a significant increase in pancreatic

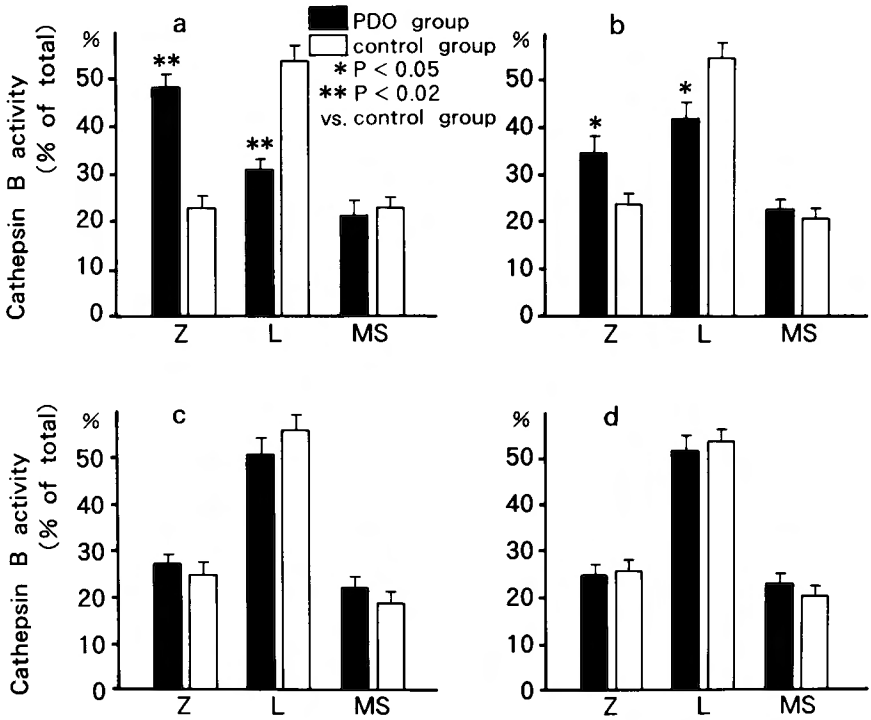

Fig. 4 Effect of PDO on changes in distribution of cathepsin B in subcellular fractionation of pancreatic acinar cells in rabbits, (a) 6 hours after PDO, (b) 12 hours after release of PDO, (c) 24 hours after release of PDO, and (d) 36 hours after release of PDO
Z: zymogen fraction (1.0 KP), L: lysosomal fraction (12 KP), MS: microsomal and soluble fraction (12 KS)
There were 6 animals in the PDO group, and 5 animals in the control group at each stage. mean±SEM.

Table 5 Effect of pancreatic duct obstruction on changes in lysosomal fragility in in-vitro incubation.

Stage	Group	n	Cathepsin B leakage (Soluble activity) (% of total) Incubation time (min)		
			30	60	90
6 hours after pancreatic duct obstruction (PDO)	PDO	6	10±2	25±3*	38±3**
	CONT	5	6±2	14±2	23±2
12 hours after removal of PDO	PDO	6	9±2	21±2	33±2*
	CONT	5	7±2	13±2	34±2
24 hours after removal of PDO	PDO	6	7±2	13±2	26±2
	CONT	5	6±2	15±2	27±2
36 hours after removal of PDO	PDO	6	8±2	16±2	25±2
	CONT	5	7±2	14±2	22±2

The leakage of cathepsin B from lysosomes is expressed as a % of total activity. *, $p<0.05$; **, $p<0.02$ compared with CONT group.

Table 6 Effect of pancreatic duct obstruction on changes in mitochondrial fragility in in-vitro incubation.

Stage	Group	n	MDH leakage (Soluble activity) (% of total) Incubation time (min)		
			30	60	90
6 hours after pancreatic duct obstruction (PDO)	PDO	6	11±2	27±3*	40±4**
	CONT	5	8±2	17±2	26±2
12 hours after removal of PDO	PDO	6	10±2	22±2	35±3*
	CONT	5	7±2	16±2	25±2
24 hours after removal of PDO	PDO	6	9±2	19±2	28±2
	CONT	5	8±2	18±2	29±2
36 hours after removal of PDO	PDO	6	7±2	15±2	24±2
	CONT	5	8±2	17±2	28±2

Soluble MDH (malate dehydrogenase) activity is expressed as a % of total activity as an index of mitochondrial fragility. *, $p<0.05$; **, $p<0.02$ compared with CONT group.

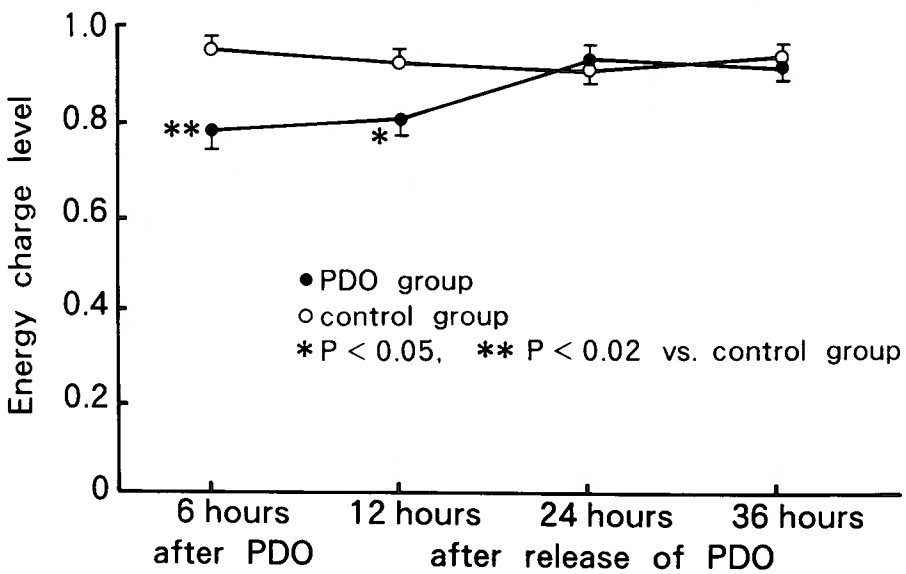

Fig. 5 Effect of PDO on changes in pancreatic energy charge level (ATP+0.5 ADP/ATP+ADP+AMP) in rabbits There were 6 animals in the PDO group and 5 animals in the control group at each stage. mean±SEM.

amylase content, suggesting congestion of pancreatic digestive enzymes during PDO, which was still present 12 hours after the release of the PDO. But at 24 hours and 36 hours, there were no significant differences between the two groups (Table IV). In regard to the pancreatic cathepsin B content, 6-hour PDO caused a slight but not significant increase in the pancreatic cathepsin B content. At 12, 24, and 36 hours there were no significant differences.

Subcellular distribution of cathepsin B

Pancreatic duct obstruction (PDO) for 6 hours caused a significant increase of cathepsin B activity in the zymogen fraction (1.0 KP) (48±3%) and a significant decrease of cathepsin B activity in the lysosomal fraction (12 KP) (31±2%); control group: 1.0 KP, 23±2%; 12 KP, 54±3%.

These changes indicate a marked shift of cathepsin B from the lysosomal fraction to the heavier zymogen fraction, indicating redistribution of cathepsin B in the subcellular fractions of acinar cells (Fig. 4a). This redistribution was still present 12 hours after the release of the PDO (1.0 KP, 35±3%; 12 KP, 42±3%); control group: 1.0 KP, 24±2%; 12 KP, 55±3% (Fig. 4b). At 24 hours and 36 hours, there were no significant differences between the PDO groups (24 hours: 1.0 KP, 27±2%; 12 KP, 51±3%; 36 hours: 1.0 KP, 25±2%; 12 KP, 52±3%) and the control group (24 hours: 1.0 KP, 25±2%; 12 KP, 56±3%; 36 hours: 1.0 KP, 26±2%; 12 KP, 54±3%), and the redistribution of cathepsin B almost disappeared (Fig. 4c, d).

Lysosomal and mitochondrial fragility in in-vitro incubation

Pancreatic duct obstruction (PDO) for 6 hours caused a significantly increased and accelerated leakage of cathepsin B from lysosomes in in-vitro incubation, especially after 60 and 90 min of incubation, compared with the control group, indicating that PDO increased and augmented lysosomal fragility. This increased lysosomal fragility continued by 12 hours after the release of the PDO, particularly after 90 min of incubation. At 24 hours and 36 hours however, increased lysosomal fragility was no longer observed (Table V).

Malate dehydrogenase (MDH) leakage from mitochondria showed the same changes, and 6-hour PDO caused accelerated leakage of MDH from mitochondria and increased mitochondrial fragility. This mitochondrial fragility was particularly marked in preparations incubated for 60, and 90 min and it continued for 12 hours after the release of the PDO, but at 24 and 36 hours mitochondrial fragility was no longer evident (Table VI).

Pancreatic adenylate energy charge levels

Pancreatic duct obstruction (PDO) for 6 hours caused a significant decrease in pancreatic adenylate energy charge levles (0.78±0.04; control S; 0.95±0.03), which persisted for 12 hours (0.80±0.03; control S: 0.92±0.02). At 24 and 36 hours there were no significant differences between the PDO group (24 hours, 0.92±0.03; 36 hours, 0.91±0.03) and the control group (24 hours, 0.90±0.03; 36 hours, 0.93±0.04), indicating recovery of pancreatic energy metabolism after PDO (Fig. 5).

Discussion

Gallstone pancreatitis in humans appears to be precipitated by the passage of a stone through or its incarceration in the terminal portion of the common bile duct[11, 24]. The mechanism whereby such a stone might precipitate acute pancreatitis has been the subject of many studies and continues to be an issue of considerable controversy. The studies reported in this communication may provide an important clue to the understanding of the events leading to pancreatitis, since they show that in the physiological state pancreatic secretagogues, such as caerulein, can stimulate the secretion of lysosomal hydrolase into pancreatic juice as they do the classical digestive enzymes, such as amylase. This finding indicates that a considerable amount of cathepsin B present in the pancreatic juice could lead to the activation of pancreatic digestive enzymes in the pancreatic duct system, since cathepsin B can activate trypsinogen, and trypsin can activate other digestive enzymes.

Generally, pancreatic digestive enzymes and lysosomal hydrolases are transported separately from the Golgi apparatus to their own subcellular compartments, condensing vacuoles and lysosomes, and theoretically there is no colocalization of these two types of enzymes in acinar cells. However, at the start of the transport of these two types of enzymes, they share a common pathway

from the ribosomes in the endoplasmic reticulum to the Golgi apparatus, and this mixture of lysosomal hydrolases and digestive enzymes seems to be accidental.

In both caerulein-induced[28, 29, 35] and diet-induced[16] pancreatitis, marked enlargement of zymogen-containing organelles in the cell apex and colocalization of lysosomal hydrolases and digestive enzymes within large acidic cytoplasmic vacuoles have been observed. In this study, too, subcellular fractionation experiments showed that duct obstruction leads to a redistribution of cathepsin B activity and that, as a result, lysosomal hydrolase becomes localized in a fraction that is rich in digestive enzymes. The colocalization of these two enzymes observed in our present study is probably the result of crinophagy; i.e. discharge of secretory granules into lysosomes and a defect in the normal sorting events by which digestive enzymes and lysosomal hydrolases are separated from each other as they pass through the Golgi apparatus[11]. This colocalization phenomenon might be an important triggering event in the evolution of pancreatitis, because the lysosomal hydrolase, cathepsin B, can activate trypsinogen, and trypsin can activate other digestive enzymes. Thus, colocalization could, under appropriate conditions, result in the intra-acinar cell activation of potentially dangerous digestive enzymes. In our present study, duct obstruction was found to cause a rise in portal serum amylase levels, lysosomal enzyme levels, microscopic mild edema, slight acinar cell vacuolization, and increased lysosomal and mitochondrial fragility. If digestive enzymes can be activated within acinar cells and injure lysosomes or mitochondria, we can rationally expect all these results. Furthermore, impaired and depressed pancreatic energy charge levels induced by pancreatic duct obstruction could be due to the mitochondrial injury induced by duct obstruction. Although duct obstruction might stimulate crinophagy and lead to the colocalization of digestive enzymes and lysosomal hydrolases by that mechanism, these observations suggest an alternative explanation: duct obstruction, possibly by inhibiting exocytosis, might interfere with the specific events which are involved in lysosomal enzyme transport from the Golgi complex to lysosomes[18, 27, 31], and thus cause lysosomal hydrolase to be diverted into the digestive enzyme pathway.

Another important finding in our present study was that the amylase and cathepsin B output in both the secretin and the caerulein fractions was augmented in the recovery stage after pancreatic duct obstruction, when the redistribution of cathepsin B in acinar cells had almost disappeared. Since short-term (6-hour) pancreatic duct obstruction caused a rise in the pancreatic amylase content, which means congestion of digestive enzymes in acinar cells during pancreatic duct obstruction, the augmented secretion of amylase and lysosomal enzymes in both the baseline (secretin) and stimulated fraction seems to play an important role in the maintenance of normal acinar cell organization during the recovery stage after pancreatic duct obstruction, eliminating the congestion of pancreatic digestive enzymes. In regard to the augmented cathepsin B output during recovery from pancreatic duct obstruction, we have no clear explanation at present, but it may be that pancreatic duct obstruction causes an increase of lysosomal enzymes in zymogen granules.

The augmented secretion of both digestive enzymes and lysosomal hydrolase into pancreatic juice and the pancreatic duct system and the redistribution of lysosomal enzyme in the pancreatic acinar cells might have special clinical importance in the etiology of gallstone pancreatitis. Gallstone attacks are often repeated, and after the first obstruction induced by a gallstone, if the secretion of pancreatic secretagogues, such as cholecystokinin and secretin, is stimulated by food intake, colocalized digestive enzymes and lysosomal hydrolases can be secreted together in greater than normal amounts into the pancreatic juice; but when the pancreatic duct system is obstructed by another stone, or another attack, or if edema of the sphincter of Oddi persists, there is no way for these

digestive enzymes and lysosomal hydrolases to drain into the pancreatic juice; in addition, within the acinar cells, there may be another redistribution of lysosomal enzymes. In the normal physiological state, a connection between the pancreatic ductal space and the peri-acinar interstitial space has been reported[2, 7], and pancreatic duct obstruction, with or without hypersecretion, seems to increase this space and to make it easier for digestive enzymes to enter into the systemic circulation or the interstitium of the pancreas. Under these conditions, the exocrine pancreas would be exposed to the activation of digestive enzymes by lysosomal hydrolase both within the acinar cells, where lysosomal hydrolases are colocalized in zymogen granules, and outside the acinar cells, where lysosomal enzymes and digestive enzymes are colocalized in the pancreatic duct system and pancreatic interstitium. These circumstances would lead to ductal hypertension and damage to the protective barrier of the ductal epithelium[17] induced by simple mechanical pressure only. The influx of infected biliary juice sometimes found in cholelithiasis would make the pancreas more susceptible to autodigestion both inside and outside the acinar cells.

In clinical acute pancreatitis and in experimental models (diet-induced, secretagogue-induced, or due to pancreatic duct obstruction), this disease seems to have a rather broad spectrum, and it is accepted that edematous pancreatitis can, under appropriate conditions such as hemorrhagic shock progress to the more severe forms of pancreatitis characterized by hemorrhage and/or necrosis of the gland[25]. Potential modulators of the severity of pancreatitis, are alterations in the pancreatic microcirculation[9], and changes in pancreatic ductal permeability induced by oral agents[36], which might permit mild pancreatitis to progress to a more severe form. Although the factors responsible for such a progress are not yet clearly understood, the secretion of lysosomal enzyme, which can potentially activate pancreatic digestive enzymes, into the pancreatic juice in bulk, when appropriately stimulated by pancreatic secretagogues, seems to favor the conversion of the mild edematous form into severe hemorrhagic and necrotic pancreatitis, and is a possible trigger of pancreatitis in the "common channel" theory.

Our pancreatic duct obstruction model provides a very mild type of pancreatitis, and this model seems to be very useful in clarifying the very early events of acute pancreatitis. We now have three different animal models of experimental pancreatitis. The mildest is our present model of short-term pancreatic duct obstruction, the second is secretagogue-induced pancreatitis, and the most severe is diet-induced pancreatitis. The three models seem to represent different degrees of severity in the broad spectrum of acute pancreatitis, but each has the same phenomenon of redistribution of lysosomal hydrolases and colocalization of lysosomal enzymes and digestive enzymes. It is very useful to have these three models to determine the factors which affect the severity of this disease. Although the currently reported studies support the hypothesis that duct obstruction may be important in the pathogenesis of gallstone pancreatitis, it is clear from these as well as many other studies that duct obstruction alone is not sufficient to cause the more definite morphological changes in the pancreas. Clearly, other events must occur if the changes induced by duct obstruction are to lead to the injuries seen in more severe pancreatitis. Studies designed to identify and clarify those events are of great importance, because they are likely to be the ultimate determinants of the severity of pancreatitis and to advance our knowledge of the pathogenesis and pathophysiology of this disease. Even more important, they may lead to therapeutic advances.

In this study, finally, we cannot explain clearly the significance of the secretion of lysosomal enzymes into pancreatic juice during stimulation by pancreatic secretagogues, but in a recent study we showed that other lysosomal enzymes, including arylsulfatase, N-acetyl-β-D-glucosaminidase, β-D-

galactosaminidase and leucine nephthylamidase are secreted into pancreatic juice during stimulation by caerulein. Although these findings strongly suggest that exocytosis of lysosomes is induced by gut hormones, their pathophysiological roles in the pancreas remain to be investigated.

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和文抄録

家兎における膵管閉塞による細胞内小器官の脆弱性

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家兎において短期間（6時間）膵管閉塞が，膵液中へのライソゾーム酸素分泌動態，ライソゾームとミトコンドリアの脆弱性におよぼす影響を検討した．6時間の膵管閉塞は，高 amylase 血症，膵消化酵素のうっ滞とともに，膵液中への amylase と cathepsin B 分泌量の減少，ライソゾームおよびミトコンドリアの脆弱性の亢進，さらに，cathepsin B の膵腺房細胞内での再分布をもたらした．これらの変化は，膵管閉塞解除後12時間持続した．逆に，24時間目には，膵液中への amylase と cathepsin B の分泌量の増加とともに，膵腺房細胞内では cathepsin B の再分布はほぼ消失した．これらの結果は，ライソゾームやミトコンドリア等の細胞内小器官の脆弱性とライソゾーム酵素の再分布が，膵管閉塞時の膵傷害の発生過程で重要な役割を果していることを示唆するとともに，この傷害の回復過程での膵液中へのライソゾーム酵素分泌の重要性をも示唆させた．