

Protective Effects of Combined Therapy with A Protease Inhibitor, ONO 3307, and A Xanthine Oxidase Inhibitor, Allopurinol on Temporary Ischaemic Model of Pancreatitis in Rats

TETSUYA HIRANO, TADAO MANABE, GAKUJI OHSHIO and YOSHINORI NIO

First Department of Surgery, Faculty of Medicine, Kyoto University, Kyoto Japan

Received for Publication, Feb. 13, 1992

Abstract

The protective effect of a new potent protease inhibitor, ONO 3307, in combination with a xanthine oxidase inhibitor, allopurinol, was tested in pancreatobiliary duct obstruction (PBDO) with temporary pancreatic ischemia in rats.

After PBDO with ischemia, we observed hyperamylasemia, pancreatic edema, congestion of amylase and lysosomal enzyme cathepsin B as well as impaired output of amylase and cathepsin B into the pancreatic juice and a redistribution of lysosomal enzyme from the lysosomal fraction to the zymogen fraction. The administration of ONO 3307 plus allopurinol almost completely prevented the pancreatic injuries induced by PBDO with ischemia.

These results indicate the important roles of temporary pancreatic ischemia in the pathogenesis of pancreatic damage and the usefulness of combination therapy with a new potent protease inhibitor and xanthine oxidase inhibitor in the protection against clinical acute pancreatitis.

Introduction

It has been suggested that many factors, such as pancreatic duct obstruction^{1,2}, hypersecretory condition³, bile-reflux^{4,5}, and ischaemia^{6,7} of the pancreas are closely related to the early events in the pathogenesis of acute pancreatitis. Moreover, redistribution of lysosomal enzyme from the lysosomal fraction to the zymogen fraction and colocalization of lysosomal enzymes with digestive enzymes have been described in the experimental pancreatitis^{1,2,3,8,9,10,11}. This colocalization of lysosomal enzymes with digestive enzymes has been suggested to play an important role in the development of pancreatic injury, because lysosomal enzyme cathepsin B can activate trypsinogen^{12,13,14}, and trypsin can activate many other key enzymes in pancreatitis. Furthermore, reports have accumulated about the protective effects of new potent synthetic protease inhibitors with relatively low molecular weights on the exocrine pancreas in various models of acute pancreatitis

Key words: Protease inhibitor, Xanthine oxidase inhibitor, Ischaemia, Redistribution of lysosomal enzyme, Cathepsin B, Pancreatic secretion of lysosomal enzyme.

索引用語: プロテアーゼインヒビター, キサンチンオキシダーゼインヒビター, 虚血, ライソゾーム酵素再分布, カテプシン B, ライソゾーム酵素の脾性分泌.

Present Address: First Department of Surgery, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyo-ku, Kyoto 606, Japan.

^{15,16,17,18,19}, and the use of such a new potent protease inhibitor may be useful in the treatment of clinical pancreatitis.

In ischaemia models of pancreatitis, the ischaemia and reperfusion relationship has been reported to play an important role in the pathogenesis of pancreatic injury through the formation of free radicals^{20,21}. It is possible that free radicals form a common pathway of injury in several forms of acute pancreatitis, because there have been several reports about the close relationship of free radicals in the other models of pancreatitis: caerulein-induced²², and choline-deficient ethionine supplemented diet-induced²³. These reports about free radicals suggest that free radical scavengers may be useful at any time in the treatment of pancreatitis.

In this study we evaluated the protective effect of combined therapy with a new potent protease inhibitor, ONO 3307 (4-sulfamoylphenyl 4-guanidinobenzoate methanesulfonate), and allopurinol against the multifactor-related pancreatic injury induced by pancreatic biliary duct obstruction in a temporary ischaemia model.

Materials and Methods

Animal preparation

A total of 64 male Wistar rats weighing about 350 g (Funahashi Farm, Shizuoka, Japan) were used in this study. They were kept in air-conditioned ($23 \pm 3^\circ\text{C}$) animal quarters and allowed to become acclimatized to standard laboratory conditions for at least 4 days. Experiments were begun after a 16-hour fast. The rats divided into the following 4 groups:

- a) Pancreatico-biliary duct obstruction (PBDO) group (12 rats)—After a 16-hour fast, general anesthesia was induced by intraperitoneal pentobarbital injection (25 mg/kg), and catheterization (V-3 catheter, Insul Tab, Woburn, MA, U.S.A.) of the superior vena cava was performed through the right external jugular vein. The catheter was tunneled beneath the skin of the back to the root of the tail and brought out. Laparotomy was performed through an upper midline incision, and the pancreatico-biliary duct (PBD) was occluded by a small metal clip just adjacent to the duodenum. Just after occlusion of the PBD, secretion ($0.2 \text{ CU/kg} \cdot \text{hr}$) (Sigma Chemical, St. Louis, MO, U.S.A.) and caerulein ($0.2 \mu\text{g/kg} \cdot \text{hr}$) (Sigma Chemical) were infused for 30 minutes with an infusion pump. At 1 hour after occlusion of the PBD, the metal clip was removed and the abdomen was closed. Animals were kept in cages for the next experiments.
- b) Ischaemia group (14 rats)—As in the PBDO group, the pancreatico-biliary duct was ligated, and secretin and caerulein were infused for 30 minutes. Then pancreatic ischaemia was induced by ligation of the caeliac and superior mesenteric arteries with the clips for next 30 minutes. After this 30-minutes pancreatic temporary ischaemia with PBDO, clips to the duct and arteries were removed, and the abdomen was closed.
- c) Pretreatment group (16 rats)—As in the ischaemia group, rats were prepared with PBDO and secretin and caerulein stimulation and ischaemia. Starting 1 hour before and continuing throughout the experiment a new potent protease inhibitor, ONO 3307, was infused in a dose of $2 \text{ mg/kg} \cdot \text{hr}$, and just before ligation of the PBD, allopurinol (Sigma Chemical) was injected intravenously in a dose of 30 mg/kg , and just after removal of the ligation of the PBD and of the arteries, allopurinol (20 mg/kg) was injected again. Thereafter, every 2 hours allopurinol was injected intravenously in a dose of 20 mg/kg for 10 hours (5 times). ONO 3307 was donated by ONO Pharmaceutical Company, Osaka, Japan.

d) Control group (10 rats)—As in the PBDO group, catheterization and laparotomy were performed and 1 hour after gentle manipulation of the pancreatico-biliary duct and a portion of the caeliac and superior mesenteric arteries, the abdomen was closed. In addition, other two groups; PBDO with caerulein and secretion stimulation and ischaemia plus only ONO 3307 group ($n=6$) and only allopurinol group ($n=6$) were used.

All the rats received heparinized (30 U/ml) lactate-Ringer solution at a rate of 0.58 ml/hr during the whole experiment (also after surgery) and were kept on heating pads at 40°C under overhead lamps, to maintain core temperature. After surgery, all the animals were fasted but given free access to tap water. Twelve hours after the removal of the ligations from the PBD and arteries, rats were sacrificed by a large dose of intravenous pentobarbital.

Serum amylase levels and pancreatic water, amylase, and cathepsin B content

The abdomen was reopened, and blood was drawn from the inferior vena cava for the determination of serum amylase levels. Portions of pancreas were removed quickly and one small portion was used for the determination of pancreatic oedema by a comparison of the weight immediately after sacrifice (wet weight) to that of the same sample after incubation at 150°C for 48 hours in a desiccator (dry weight). Other small portions of the pancreas were homogenized in 5 ml ice-chilled phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100 (Fisher Scientific) in a Brinkmann Polytron (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.) for the measurement of the pancreatic content of amylase and cathepsin B, as a lysosomal enzyme. Both amylase and cathepsin B activity, as well as deoxyribonucleic acid (DNA) concentration, were measured in the resulting supernatant after low speed centrifugation ($150 \times g$, 15 min, 4°C) and were expressed as U/mg DNA.

Histological changes

Other very small portions of pancreas from each group were fixed overnight in phosphate-buffered (pH 7.4) 10% neutral formalin solution. After paraffin embedding, sectioning and staining with hematoxylin-eosin, the sections were examined by a blinded observer, and intestinal edema, acinar cell vacuolization, inflammatory cell infiltration, and acinar cell necrosis were graded on a 0–4+ scale (0, no change; 4+, maximum change).

Subcellular distribution of amylase and cathepsin B activity

The remaining portions of the pancreas were homogenized in 6 ml of ice-chilled 5 mM MOPS (3-(N-morpholino) propanesulfonic acid) (Sigma Chemical) buffer (pH 6.5) containing 1 mM $MgSO_4$ and 250 mM sucrose by 3 up-and-down strokes of a Dounce homogenizer (Wheaton, Millville, NJ, U.S.A.) in a cold room. Unbroken cells and debris were removed by low speed centrifugation ($150 \times g$, 15 min, 4°C). The resulting supernatant was considered to be the entire sample for later calculations and to contain 100% of all measured components. Subcellular fractionations were performed by differential centrifugation, as described by Tartakoff and Jamieson²⁴, with minor modifications for studies of rat tissue²⁵. Briefly, the supernatant described above was centrifuged ($1300 \times g$, 15 min 4°C) to yield a “zymogen granule” pellet and another supernatant. This supernatant was centrifuged ($12,000 \times g$, 12 min, 4°C) to yield a “combined lysosomal and mitochondrial” pellet and a supernatant, considered to be the “microsomal and soluble” fraction. The various pellets obtained during fractionations were resuspended individually in 2 ml of ice-chilled 5 mM MOPS buffer, and the amylase and cathepsin B activity in each fraction was measured and expressed as a percentage of the total activity as an index of distribution of both digestive and lysosomal enzymes in the pancreatic acinar cells.

In-vivo amylase and cathepsin B output

Other new rats were used in this *in-vivo* experiment. For each group, at selected time, animals were anesthetized with intravenous pentobarbital (25 mg/kg) and the abdomen was reopened. After the formation of an external biliary fistula (PE 10, Clay Adams, Parsippany, NJ, U.S.A.) at the liver hilum, the pancreatico-biliary duct was cannulated (PE 50, Clay Adams) just adjacent to the duodenum. After 30 minutes for stabilization, caerulein was infused in a dose of $0.2 \mu\text{g/kg} \cdot \text{hr}$ for 1 hour for stimulation of pancreatic secretion, and pancreatic juice was collected in pre-weighed eppendorf tubes on ice. The volume of pancreatic juice, and amylase and cathepsin B activities were measured. Both amylase and cathepsin B outputs were expressed as U/kg $\cdot$ hr.

Assays

Amylase activity was measured by the method of Bernfeld²⁶ with soluble starch as the substrate, and cathepsin B activity was measured fluorometrically by the method of McDonald and Ellis²⁷ with CBZ-arginyl-arginine- β -naphthylamide (Bachem Bioscience, Philadelphia, PA, U.S.A.) as the substrate. Deoxyribonucleic acid (DNA) concentration was measured fluorometrically by the method of Labarca and Paigen²⁸, with calf thymus DNA (Sigma Chemical) as the standard.

Statistical analysis

This results reported in this study represent the mean $\pm$ SEM for n determinations. Differences between groups were evaluated by ANOVA and Tukey method. For evaluating the histological changes, Wilcoxon rank-sum test was used and significant differences were defined as those associated with a probability value (p) of less than 0.05 ($p < 0.05$).

Results

All the animals in the PBDO and ischaemia groups as well as those in the treatment group survived after surgery.

Serum amylase levels, pancreatic water, amylase and cathepsin B content

One hour pancreatico-biliary duct obstruction (PBDO) with 30 minutes of stimulation by secretion ($0.2 \text{ CU/kg} \cdot \text{hr}$) and caerulein ($0.2 \mu\text{g/kg} \cdot \text{hr}$) plus 30 minutes of temporary pancreatic ischaemia caused a significant increase in serum amylase levels, pancreatic water, amylase and cathepsin B content, but PBDO with secretion and caerulein stimulation without ischaemia did not

Table 1 Effects of ONO 3307 and allopurinol on the changes in serum amylase levels, pancreatic water, amylase and cathepsin B content in rat pancreatico-biliary duct obstruction (PBDO) with pancreatic ischaemia

Group	n	Serum amylase levels (U/ml)	Pancreatic water content (% of wet weight)	Pancreatic amylase content (U/mg DNA)	Pancreatic cathepsin B content (U/mg DNA)
PBDO	6	$8 \pm 2^{**}$	77 ± 2	$573 \pm 62^*$	$1226 \pm 138^*$
Ischaemia	7	23 ± 3	81 ± 2	728 ± 54	1724 ± 189
Pretreatment	8	$9 \pm 2^{**}$	$76 \pm 2^*$	$481 \pm 57^{**}$	$1275 \pm 153^*$
Control	5	$6 \pm 1^{***}$	$74 \pm 1^{**}$	$439 \pm 45^{***}$	$1182 \pm 126^{**}$
ONO 3307	6	$14 \pm 2^*$	78 ± 2	$592 \pm 48^*$	1347 ± 165
Allopurinol	6	16 ± 2	79 ± 2	604 ± 51	1423 ± 154

PBDO: pancreatico-biliary duct obstruction, Ischaemia: PBDO with pancreatic ischaemia, Pretreatment: PBDO with pancreatic ischaemia plus ONO 3307 and allopurinol administration, Control: controlled laparotomy groups, ONO 3307: PBDO with pancreatic ischaemia plus only ONO 3307 group, Allopurinol: PBDO with pancreatic ischaemia plus only allopurinol group, $^*p < 0.05$, $^{**}p < 0.02$, and $^{***}p < 0.01$ compared with ischaemia group.

Table 2 Effects of ONO 3307 and allopurinol on the changes in pancreatic acinar cell histology in rat PBDO with ischaemia

Group	n	Histological changes of pancreatic acinar cells			
		Interstitial edema	Acinar cell vacuolization	Inflammatory cell infiltration	Acinar cell necrosis
PBDO	6	0.3±0.2* (0-1)	0.3±0.2* (0-1)	0.2±0.2* (0-1)	0.2±0.2* (0-1)
Ischaemia	7	2.1±0.3 (1-3)	2.4±0.2 (2-3)	2.3±0.3 (1-3)	1.9±0.3 (1-3)
Pretreatment	8	0.4±0.2* (0-1) 0**	0.3±0.2* (0-1) 0**	0.3±0.2* (0-1) 0**	0.2±0.2* (0-1) 0**
Control	5	(0)	(0)	(0)	(0)
ONO 3307	6	1.2±0.2 (1-2)	1.2±0.2* (1-2)	1.2±0.4 (0-2)	1.2±0.2 (1-2)
Allopurinol	6	1.4±0.2 (1-2)	1.2±0.2* (1-2)	1.4±0.4 (1-3)	1.4±0.4 (1-2)

Histological changes were blindly graded on a scale from 0 (no changes) to 4+ (maximum changes and the values are expressed as means±SEM, ()); the ranges of the values. The symbols in the groups are the same as in Table 1.

*p<0.05, **p<0.02 compared with ischaemia group by Wilcoxon rank-sum test.

cause significant changes in these parameters. The administration of ONO 3307 and allopurinol had a significant protective effect against the hyperamylasemia, pancreatic oedema, and congestion of pancreatic digestive and lysosomal enzyme in acinar cells induced by PBDO with hypersecretion plus temporary pancreatic ischaemia. The administration of only ONO 3307 also had a significant protective effect against the hyperamylasemia and congestion of pancreatic amylase, but less than the combination administration. The administration of only allopurinol also had a significant protective effect against the hyperamylasemia, but less than the combined administration (Table 1).

Histological changes

PBDO with hypersecretion plus temporary pancreatic ischaemia caused moderate but significant histological changes: interstitial oedema, acinar cell vacuolization, inflammatory cell infiltration, and acinar cell necrosis. PBDO with hypersecretion induced by secretion and caerulein without ischaemia caused only very mild histological changes. The administration of ONO 3307 in combination with allopurinol almost completely prevented the acinar cell damage induced by PBDO with ischaemia. The administration of only ONO 3307 or allopurinol also had a significant protective effect against the histological changes only in the acinar cell vacuolization (Table 2).

Subcellular distribution of amylase and cathepsin B activity

One hour of PBDO with 30 minutes of stimulation with secretion and caerulein plus 30 minutes of ischaemia caused a significant decrease of amylase activity in the zymogen granule fraction and a significant increase in the microsomal and soluble fraction. PBDO without ischaemia caused no significant changes. The administration of ONO 3307 in combination with allopurinol had a significant protective effect against the increased fragility of zymogen granules induced by PBDO with ischaemia. PBDO with ischaemia also significantly increased cathepsin B activity in zymogen granules and significantly decreased it in the lysosomal fraction, indicating a redistribution of lysosomal enzyme from the lysosomal to the heavier zymogen fraction and a colocalization of lysosomal enzyme with pancreatic digestive enzyme. PBDO without ischaemia caused no significant changes in cathepsin B activity distribution. The administration of ONO 3307 in combination

Table 3 Effects of ONO 3307 and allopurinol on the changes in pancreatic subcellular distribution of amylase and cathepsin B activity in rat PBDO with ischaemia

Group			Fractions (% of total)		
			Zymogen	Lysosomal	Myrosomal and soluble
Amylase activity	PBDO	6	39±2**	17±2	44±2*
	Ischaemia	7	31±2	16±2	53±3
	Pretreatment	8	37±2*	17±2	46±2*
	Control	5	40±2**	18±2	42±2**
	ONO 3307	6	35±5	18±2	47±3
	Allopurinol	6	34±2	17±2	49±2
Cathepsin B activity	PBDO	6	27±2**	57±2**	20±2
	Ischaemia	7	38±3	40±3	22±2
	Pretreatment	8	28±2***	54±2**	18±2
	Control	5	24±2***	59±2***	17±2
	ONO 3307	6	31±2	50±2	19±2
	Allopurinol	6	34±3	46±3	20±2

*p<0.05, **p<0.02, and ***p<0.01 compared with ischaemia group

with allopurinol significantly inhibited the redistribution of cathepsin B in acinar cell induced by PBDO with ischaemia. The administration of only ONO 3307 or allopurinol had no significant changes in the distribution of cathepsin B and amylase in acinar cells (Table 3).

In-vivo amylase and cathepsin B output

In the ischaemia group pancreatic exocrine functions stimulated by caerulein (0.2 µg/kg · hr) were significantly lower than in the control group. Pancreatic juice volume after caerulein stimulation was significantly lower than in the control group. PBDO without ischaemia caused only a slight, but not-significant reduction. The administration of ONO 3307 with allopurinol had a significantly protective effect against the reduction in pancreatic juice volume induced by PBDO with ischaemia. Both amylase and cathepsin B outputs showed similar changes, and 12 hours after PBDO with ischaemia, both amylase and cathepsin B outputs after caerulein stimulation were significantly lower than in the control group. PBDO without ischaemia only a slight, not-significant decrease in amylase and cathepsin B output. Administration of ONO 3307 in combination with allopurinol significantly improved both amylase and cathepsin B outputs after caerulein stimulation (Table 4).

Table 4 Effects of ONO 3307 and allopurinol on the changes in caerulein induced pancreatic juice volume, amylase and cathepsin B output in rat PBDO with ischaemia

Group	n	Juice volume (ml/kg/hr)	Amylase output (U/kg/hr)	Cathepsin B output (U/kg/hr)
PBDO	6	1.58±0.21*	7335±843*	20±2*
Ischaemia	7	0.61±0.17	2889±328	7±3
Pretreatment	8	1.48±0.23*	7127±946*	18±3*
Control	5	1.72±0.19**	8724±921**	23±2**

*p<0.02 and **p<0.01 compared with ischaemia group.

Discussion

In this present study, we evaluated the effects of short-term pancreatobiliary duct obstruction and stimulation with secretion and caerulein plus temporary pancreatic ischaemia on the exocrine pancreas in rats, and the protective effect of a new potent synthetic protease inhibitor, ONO 3307, in combination with a well-known xanthine oxidase inhibitor, allopurinol. Short-term PBDO without ischaemia did not cause significant changes in any of the parameters examined in this study, but if ischaemia was superimposed on PBDO, pancreatic exocrine functions were significantly and markedly impaired. Short-term PBDO alone, or ischaemia alone also caused no significant changes in any of the parameters (data not shown). Moreover, PBDO with ischaemia caused a marked redistribution of lysosomal enzyme from the lysosomal fraction to the heavier zymogen fraction, indicating colocalization of lysosomal enzymes with digestive enzymes in the same subcellular compartment in acinar cells. This redistribution of lysosomal enzymes has also been described in other experimental models of pancreatitis: caerulein-induced^{8,9}, pancreatic duct obstruction¹⁻³ and diet-induced^{10,11}. Thus, this redistribution phenomenon seems to be an important triggering event in the development of acute pancreatitis. In a recent study, we noted the secretion of lysosomal enzymes into pancreatic juice stimulated by pancreatic secretagogues^{2,29}. This secretion of lysosomal enzyme into pancreatic juice seems to be closely related to changes in the transport and distribution of lysosomal enzyme in pancreatic acinar cells. PBDO with ischaemia also caused marked impairment of both cathepsin B and amylase secretion during stimulation by caerulein which suggests a disturbance in the sorting of lysosomal enzymes in acinar cells.

Pancreatic ischaemia, such as that caused by induced hypotension, has been reported to affect the severity of pancreatitis³⁰, but in the ischaemia models of pancreatitis, the production of oxygen-induced free radicals seems to be more responsible for the injury produced by ischaemia-reperfusion.

In the ischaemia-reperfusion pancreatitis models, xanthine oxidase has been reported to be the source of superoxide radicals^{30,31,32,33}, because xanthine oxidase is a ubiquitous enzyme in the gastrointestinal tract and the pancreas also has considerable amounts of this enzyme.

We tested the protective effect of allopurinol, a specific xanthine oxidase inhibitor, in the ischaemia-reperfusion model of pancreatitis superimposed on short-term pancreatic duct obstruction. ONO 3307 is a guanidino acid ester. Such new potent synthetic protease inhibitors have been reported to inhibit strongly many key pancreatic enzymes, such as trypsin, elastase, phospholipase A₂, thrombin, and kallikrein^{33,34}. Moreover, its relatively low molecular weight (430 daltons) should help it to penetrate into the acinar cells, so we were encouraged to evaluate its protective effects in this models. As in other previous studies and our own recent study^{15,16,17,18,19}, ONO 3307 showed protective effects against pancreatic injury. In this ischaemia-reperfusion model we tested it in combination with the xanthine oxidase inhibitor, allopurinol.

Our present study strongly suggests that temporary pancreatic ischaemia and oxygen-derived free radicals generated by xanthine oxidase play crucial roles in the pathogenesis and development of acute pancreatitis, and if ischaemia is superimposed on pancreatobiliary duct obstruction, the pancreatic damage would be expected to be more severe. The present study also suggest that this new potent synthetic protease inhibitor in combination with the xanthine oxidase inhibitor, allopurinol, might be of use in the protection for clinical pancreatitis, although, in this study, the agents were administered before the induction of acute pancreatitis rather than subsequently.

Finally, because many factors, such as pancreatobiliary duct obstruction, intraductal

hypertension, and pancreatic ischaemia seem to be closely related to the pathophysiology of pancreatitis, this present model of PBDO with ischaemia seems to be an aid and of benefit in the clarification of the early events of the pathogenesis of acute pancreatitis as well as possible mechanisms and the protective effects of various agents, because in its several aspects it represents moderately severe pancreatitis.

Acknowledgments

This study was supported by a grant, Scientific Research B-03454319, from the Ministry of Education, Science and Culture, and a grant from the Ministry of Health and Welfare of Japan. The authors thank Mr. Kimiko Hirano for preparing the manuscript and Ms. Yoko Manabe for typing the manuscript.

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和文抄録

ラットでの短期間総膵胆管結紮，膵管内圧上昇と 膵虚血再還流モデルにおける xanthine oxidase inhibitor と protease inhibitor の併用療法

京都大学 医学部 第一外科教室

平野 鉄也，真辺 忠夫，大塩 学而，仁尾 義則

ラットにて短期間（1時間）の総膵胆管結紮，caerulein ($0.2 \mu\text{g}/\text{kg} \cdot \text{hr}$) と secretin ($0.2 \text{ CU}/\text{kg} \cdot \text{hr}$) による膵外分泌刺激および腹腔動脈と上腸間膜動脈結紮（30分後再還流）による膵炎モデルにて xanthine oxidase inhibitor である allopurinol と protease inhibitor である ONO 3307 の併用療法を検討した。上記モデルにて，高 amylase 血症，膵浮腫，膵消化酵素の膵内うっ滞，膵腺房細胞の空胞化等の組織学的変化，さらに，細胞分画法にては，膵ライソゾーム酵素のライソゾーム分画よりチモーゲン分画への移動・再分布が観

察された。しかし，allopurinol と ONO 3307 の併用療法にてこれらすべての parameter で有意な膵保護効果が観察されたが³，allopurinol と ONO 3307 の単独療法では一部の parameter にのみ保護効果を示したのみであった。これらの結果は，本モデルにおいては，xanthine oxidase を介した oxygen-derived free radical や何らかの protease activity が，その膵損傷の発生に重要な役割を果していることとともに，臨床上での膵炎治療においても，その作用機序が異なった種々の agent の併用療法の有用性をも示唆させるものであった。