
原 著

Improved Survival in Mice with Diet-Induced Pancreatitis Treated
with New Potent Protease Inhibitor, E-3123 and
a Broad Spectrum Antibiotic, Cefmetazole

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Abstract

This study was designed to evaluate the infectious factor in the pathogenesis of acute pancreatitis and the effects of a combination therapy with a new potent protease inhibitor, E-3123, and a broad spectrum antibiotic cefmetazole (CMZ) in mice with CDE diet-induced severe acute pancreatitis. Combination therapy with E-3123 and CMZ showed significant protective effects against the high mortality rate, increased serum amylase and ascitic fluid amylase levels, pancreatic amylase and lysosomal enzyme content, plasma endotoxin levels, redistribution of lysosomal enzyme from the lysosomal to the zymogen fraction, lysosomal and mitochondrial fragility, and also improved the histological findings when compared with the E-3123 alone. These results suggest that infectious factors play an important role in the development of severe acute pancreatitis and that protease inhibitors in combination with antibiotics may be clinically beneficial.

Introduction

It is well known that hemorrhagic and necrotizing pancreatitis can be induced by feeding young female mice a choline-deficient ethionine-supplemented (CDE) diet¹⁰, and this well-established experimental model of severe acute pancreatitis is useful in improving our understanding of the pathogenesis of this disease. Infectious factors have been reported to affect the survival rate in clinically severe pancreatitis^{1, 11, 12, 13}. Furthermore, in this CDE diet-induced pancreatitis, redistribution of lysosomal enzyme from the lysosomal fraction to the zymogen fraction and colocalization of lysosomal enzyme with digestive enzymes have also been reported^{7, 15}, and lysosomal enzymes seem to play important roles in the development of acute pancreatitis^{20, 21}, because the lysosomal enzyme cathepsin B can activate trypsinogen^{5, 17} and trypsin can activate many other

Key words: Amylase, Lysosomal enzyme, Cathepsin B, Protease inhibitor, E-3123, Cefmetazole, Lysosomal fragility, Mitochondrial fragility, Redistribution of cathepsin B, CDE diet-induced pancreatitis.

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digestive enzymes. A new potent synthetic protease inhibitor, E-3123 (4-(2-succinimidoethylthio) phenyl 4-guanidinobenzoate methanesulfonate) has been reported to inhibit several key pancreatic enzymes, including trypsin and phospholipase A₂⁶, and recently there have been a few reports on the protective effects of such potent synthetic protease inhibitors in experimental pancreatitis^{9, 15, 23}.

Thus, in the treatment of acute pancreatitis, therapy against both intrapancreatic acinar cell activation of digestive enzymes and infectious factors seems to be essential in protecting the exocrine pancreas. In this study, we evaluated the protective effects of a combination therapy of protease inhibitor and antibiotics in mice with CDE diet-induced severe pancreatitis, using various parameters, including mortality rate, serum amylase levels, and subcellular organellar fragility.

Materials and Methods

Four hundreds fifty young female CD-1 mice (12–14 g) (Shizuoka Experimental Animal, Shizuoka, Japan) were used in this experiment. After an initial 24-hour fast, they were fed a choline-deficient diet enriched with 0.5% DL-ethionine (CDE) diet (U.S. Biochemical Corp., Cleveland, OH, U.S.A.) for 24 hours. They were again fasted for 24 hours, then fed a regular laboratory diet and tap water at libitum for the following 3 days. Up to 20 mice were kept in each cage. All the mice were fed thus CDE diet and divided into the following groups: a) Control animals (CONT group)—Only 0.2 ml of saline was injected subcutaneously every 6 hours. b) E-3123 treated animals (E group)—for 24 hours before the CDE diet and 5 days after the beginning of the CDE diet, E-3123 (10 mg/kg every 6 hours) was injected subcutaneously in 0.2 ml of saline. c) E-3123 plus cefmetazole (CMZ) treated animals (CMZ group)—E-3123 (10 mg/kg every 6 hours) and CMZ (50 mg/kg every 12 hours) were injected subcutaneously as in E group. In addition to these 3 groups, normal mice were also used as true controls.

For 30 mice in each group, survival rates were recorded up to 5 days after completion of the CDE diet.

At 24 hours and 48 hours after completion of the CDE diet, surviving animals (CONT group: $n=12$, E group: $n=14$, CMZ group: $n=16$) were killed by cervical dislocation, blood was drawn by heart puncture, and serum amylase levels were determined. The abdomen was opened, ascitic fluid was withdrawn through a plastic syringe, and the amylase level was determined.

Endotoxin levels were measured in the same blood samples. Blood was centrifuged ($3000 \times g$, 10 min, 4°C) and the plasma endotoxin level was measured with quantitative Chromogenic Limulus Amebocyte Lysate Assay Kits (QCL-1000, Bioproducts, MA, U.S.A.) with the endotoxin from *Escherichia coli* 0127: 138 (Worthington Biochemical, Freehold, NJ, U.S.A.) as the standard.

Portions of the pancreas were then quickly removed and homogenized in 4 ml of ice-chilled phosphate-buffered saline (pH 7.4) in a Polytron Homogenizer (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.). After low speed centrifugation ($150 \times g$, 15 min, 4°C), amylase activity and cathepsin B activity in the resulting supernatant were measured; deoxyribonucleic acid (DNA) concentration was also measured. Both amylase and cathepsin B activity in pancreatic tissue were expressed as U/mg $\cdot$ DNA.

Other small portions of the pancreas were fixed by immersion in phosphate-buffered (pH 7.4) 10% neutral formalin. After paraffin embedding, sectioning, and staining with hematoxylin-eosin, the sections were examined light microscopically by a blinded observer who recorded acinar cell changes.

At 24 and 48 hours, in each group, other new animals were sacrificed by cervical dislocation and portions of the pancreas were removed quickly. Portions of pancreases from 3 mice were used in each experiment. The trimmed and homogenized specimens were separated into various subcellular fractions by differential centrifugation by the method of Tartakoff and Jamieson²² modified to permit optimum separation of mouse pancreatic cell fractions⁴. Briefly, the pancreatic tissues were excised and homogenized in 6 ml of ice-chilled 5 mM MOPS buffer (pH 6.5) (3-(N-morpholino) propanesulfonic acid) (Sigma Chemical, St. Louis, MO, U.S.A.) containing 1 mM MgSO_4 and 250 mM sucrose. The resulting homogenate was centrifuged ($150 \times g$, 10 min 4°C). The supernatant was centrifuged ($1300 \times g$, 15 min, 4°C) to obtain a zymogen granule-rich pellet and a supernatant which was centrifuged ($12000 \times g$, 12 min, 4°C) to yield a lysosome and mitochondria-rich pellet and a supernatant, which was considered to be the microsomal and soluble fraction. The various pellets obtained during fractionation were resuspended individually in 2 ml of ice-chilled 5 mM MOPS buffer, and the cathepsin B activity was measured and expressed as a percentage of the total activity.

Using other new 3 animals in each group at each stage we performed in-vitro incubation experiments of lysosomes and mitochondria. The excised and trimmed pancreatic tissues were homogenized and subcellularly fractionated as described above. This lysosomal and mitochondrial fraction was resuspended in the same 5 mM MOPS buffer and incubated for various intervals, (30, 60, and 90 min) at 25°C in a shaking water bath in room air. The samples were then recentrifuged ($12000 \times g$, 12 min, 4°C) to separate the particulate from the soluble lysosomal and mitochondrial enzyme activities, each of which was individually measured after the separation of the pellet and supernatant. Cathepsin B activity as a lysosomal enzyme and malate dehydrogenase (MDH) activity as a mitochondrial enzyme were measured in both the pelleted and the soluble fractions. Centrifugation and subsequent measurement of particulate and soluble lysosomal and mitochondrial enzyme activities identified the rate and the extent of *in-vitro* rupture of lysosomal enzyme and mitochondrial enzyme-containing organelles. Both soluble cathepsin B and MDH activity, expressed as percentages of the total activity, were used as indexes of lysosomal and mitochondrial fragility.

Amylase activity was measured with soluble starch (Sigma Chemical) as the substrate by the method of Bernfeld³, 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 Ellis¹⁴ with CBZ-arginyl-arginine- β -naphthylamide (Bachem Bioscience, Philadelphia, PA, U.S.A.) as the substrate, and one unit (U) of activity was defined as that which releases 1 nanomole of β -naphthylamine (Sigma Chemical) per min at 38°C . Deoxyribonucleic acid (DNA) concentration was measured fluorometrically by the method of LaBarca and Paigen⁸ with calf thymus DNA (Sigma Chemical) as the standard; malate dehydrogenase (MDH) activity was measured spectrometrically by the method of Bergmeyer².

E-3123 was kindly donated by Eisai Pharmaceutical Company, Tokyo, Japan, and cefmetazole (CMZ) was purchased from Sankyo Pharmaceutical, Tokyo, Japan.

The results reported in this communication represent the means $\pm$ SEM for *n* determinations. Differences between groups were evaluated by Student's *t*-test or Wilcoxon's rank-sum test, and significant differences were defined as those associated with a probability value (*p*) of less than 0.05. For the analysis of the mortality rate, χ^2 test with Yate's correction was used.

Results

In mice the CDE diet caused severe pancreatitis with a high mortality rate (only 57% survival at 3 days, 43% at 4 days, 30% at 5 days). The administration of E-3123 significantly reduced these high mortality rates (87% survival at 3 days, 76% at 4 days, 60% at 5 days). The combination of E-3123 and CMZ had an even greater protective effect (93% survival at 3 days, 87% at 4 days, 77% at 5 days) (Fig. 1).

The CDE diet caused a significant increase in serum amylase levels (24 hours, 28 ± 3 U/ml; 48 hours, 21 ± 3 U/ml) and ascitic fluid amylase levels (24 hours, 122 ± 6 U/ml; 48 hours, 109 ± 13 U/ml vs control value of 6 ± 1 U/ml). The administration of E-3123 significantly reduced the changes induced by the CDE diet (serum amylase levels: 24 hours, 11 ± 2 U/ml; 48 hours, 9 ± 2 U/ml; ascitic fluid amylase levels: 24 hours, 43 ± 8 U/ml; 48 hours, 38 ± 9 U/ml). The combination therapy, E-3123 plus CMZ, had a slightly more protective effect than E-3123 alone (serum amylase levels: 24 hours, 10 ± 2 U/ml; 48 hours, 7 ± 2 U/ml; ascitic fluid amylase levels: 24 hours, 35 ± 6 U/ml; 48 hours, 33 ± 8 U/ml). (Fig. 2).

The CDE diet greatly increased plasma endotoxin levels (24 hours, 0.19 ± 0.05 ng/ml; 48 hours, 0.29 ± 0.07 ng/ml). In the E-3123-treated group the respective levels were 0.10 ± 0.04 ng/ml and 0.13 ± 0.05 ng/ml, and in those treated with both E-3123 and CMZ they were 0.07 ± 0.03 ng/ml and 0.09 ± 0.05 ng/ml (Fig. 3).

The CDE diet greatly increased both the amylase and the cathepsin B content of the pancreas, but as 48 hours those values were lower than at 24 hours. E-3123 injection significantly lowered

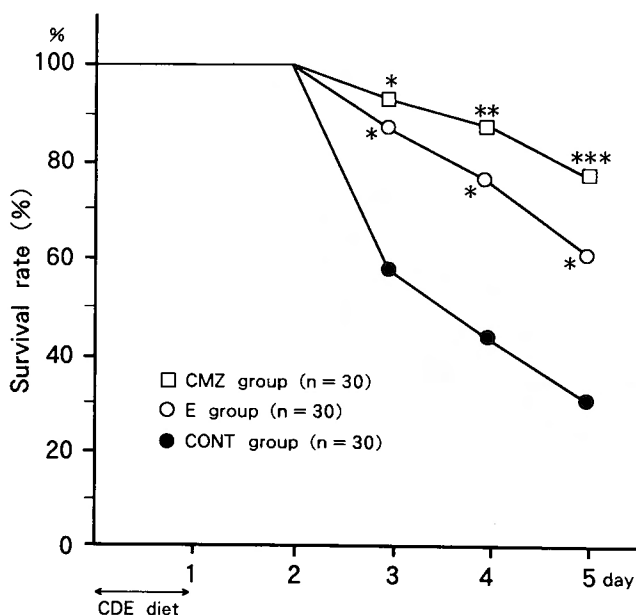


Fig. 1 Effects of E-3123 and CMZ on the survival rate of mice with CDE diet-induced pancreatitis. CONT: control group, mice were fed the CDE diet and only saline injection. E group: CDE diet with E-3123 injection; CMZ group: CDE diet with E-3123 plus CMZ injection. *, $p < 0.02$, compared with CONT group; **, $p < 0.01$ compared with CONT group and $p < 0.05$ compared with E group; ***, $p < 0.01$ compared with CONT group and $p < 0.02$ compared with E group. Mean $\pm$ SEM.

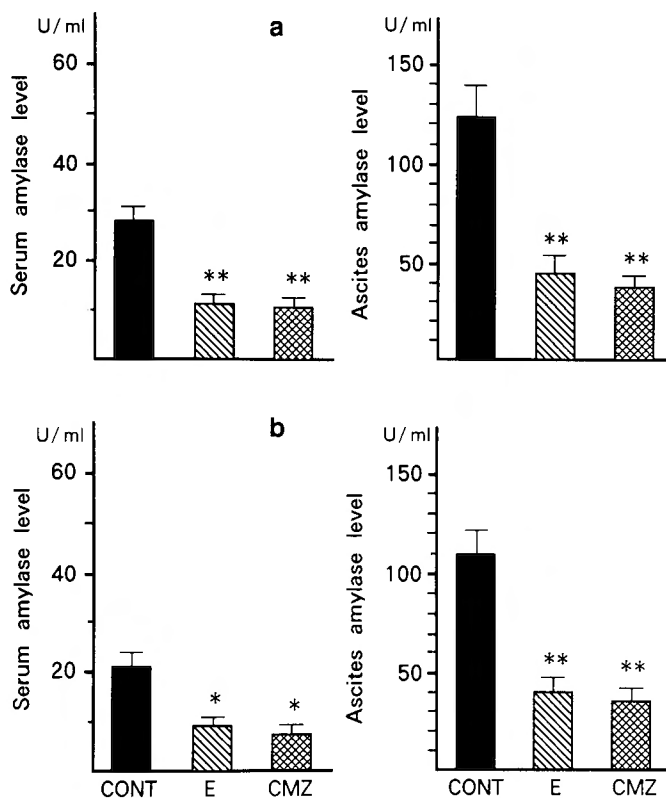


Fig. 2 Effects of E-3123 and CMZ on changes in serum amylase and ascitic fluid amylase levels at 24 (a) and 48 hours (b) after completion of CDE diet in mice. CONT group, $n=6$; E group, $n=7$; CMZ group, $n=8$; *, $p<0.05$; **, $p<0.02$, compared with CONT group; serum amylase levels of normal mice, 6 ± 1 U/ml. Mean $\pm$ SEM.

CDE diet-induced congestion of both amylase and cathepsin B in acinar cells, and the combination therapy with both E-3123 and CMZ had an even greater protective effect (Table 1).

The CDE diet caused marked histological changes in the acinar cells: vacuolization, interstitial edema, inflammatory cell infiltration, and necrosis. E-3123 had some protective effect against all these abnormalities, but it was not significant. On the other hand, the combination of E-3123 and CMZ therapy had significant protective effects against almost all these histological abnormalities (Table 2).

The CDE diet caused a significant increase of cathepsin B activity in the zymogen fraction (24 hours, $48 \pm 3\%$; 48 hours, $52 \pm 4\%$) and a significant decrease in the lysosomal fraction (24 hours, $29 \pm 2\%$; 48 hours, $26 \pm 2\%$). In normal mice zymogen fraction was $26 \pm 3\%$ and lysosomal fraction $54 \pm 3\%$. These changes reflect redistribution of cathepsin B from the lysosomal fraction to the zymogen fraction and colocalization of lysosomal enzyme and digestive enzymes. E-3123 had a significant protective effect against the redistribution of lysosomal enzyme (24 hours: zymogen fraction $35 \pm 3\%$; lysosomal fraction, $45 \pm 3\%$; 48 hours: zymogen fraction, $39 \pm 3\%$; lysosomal fraction, $42 \pm 3\%$) and E-3123 plus CMZ provided even more protection (24 hours: zymogen fraction, $30 \pm 3\%$; lysosomal fraction, $51 \pm 3\%$; 48 hours: zymogen fraction, $34 \pm 2\%$; lysosomal fraction

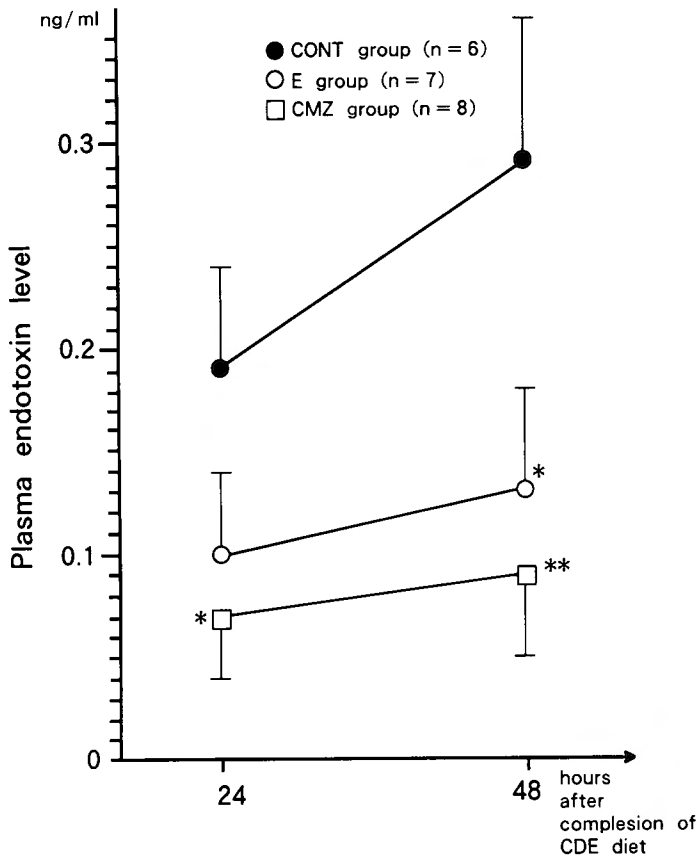


Fig. 3 Effects of E-3123 and CMZ on plasma endotoxin levels in mice with CDE diet-induced pancreatitis. *, $p<0.05$ and **, $p<0.02$ compared with CONT group. Mean $\pm$ SEM.

45 $\pm$ 3%) (Fig. 4a, b).

The CDE diet caused an accelerated and increased fragility of lysosomes in vitro, particularly if the incubation time was prolonged (≥ 60 min). These changes show the increased lysosomal fragility induced by the CDE diet. The administration of E-3123 had a significantly protective effect

Table 1 Effects of E-3123 and CMZ on the changes in pancreatic amylase and cathepsin B content in mouse CDE diet induced pancreatitis at 24 and 48 hours after completion of CDE diet.

Group	n	Pancreatic amylase content (U/mg·DNA)		Pancreatic cathepsin B content (U/mg·DNA)	
		24 hours	48 hours	24 hours	48 hours
CONT	6	958 $\pm$ 94	874 $\pm$ 92	2427 $\pm$ 184	2236 $\pm$ 175
E	7	627 $\pm$ 49**	596 $\pm$ 67*	1617 $\pm$ 198*	1479 $\pm$ 156*
CMZ	8	496 $\pm$ 68***	472 $\pm$ 53***	1428 $\pm$ 171**	1291 $\pm$ 136**

CONT; CDE diet plus saline injected group, E; CDE diet with E-3123 administration group, CMZ; CDE diet with E-3123 plus CMZ administration group, *, $p<0.01$, **, $p<0.02$, ***, $p<0.01$ compared with CONT group. Normal mice; 453 $\pm$ 44 U/mg·DNA (n=5) Mean $\pm$ SEM.

Table 2 Effects of E-3123 and CMZ on the changes in pancreatic histological changes

Group		n	Acinar cell vacuolization	Interstitial edema	Inflammatory cell infiltration	Necrosis
CONT	24 hours	6	2.7±0.2 (2-3)	2.0±0.3 (1-3)	2.3±0.3 (1-3)	2.2±0.3 (1-3)
	48 hours	6	3.7±0.2 (3-4)	2.3±0.3 (2-3)	3.2±0.3 (2-4)	2.8±0.2 (2-3)
E	24 hours	7	2.4±0.2 (2-3)	1.4±0.3 (1-3)	1.4±0.3 (1-2)	1.4±0.3 (1-2)
	48 hours	7	2.9±0.3 (2-4)	2.4±0.2 (2-3)	2.1±0.3 (1-3)	2.3±0.4 (1-3)
CMZ	24 hours	8	1.4±0.2* (1-2)	1.3±0.2 (1-2)	1.1±0.1* (1-2)	1.3±0.2 (1-2)
	48 hours	8	2.0±0.3* (1-3)	1.4±0.3 (1-3)	1.4±0.2* (1-2)	1.4±0.3* (1-3)

Histological changes were blindly graded on a scale from 0 (no changes) to 4+ (maximum changes) and the values were expressed as the means±SEM. (); the range of the values, *; $p < 0.05$ compared with CONT group with Wilcoxon rank-sum test.

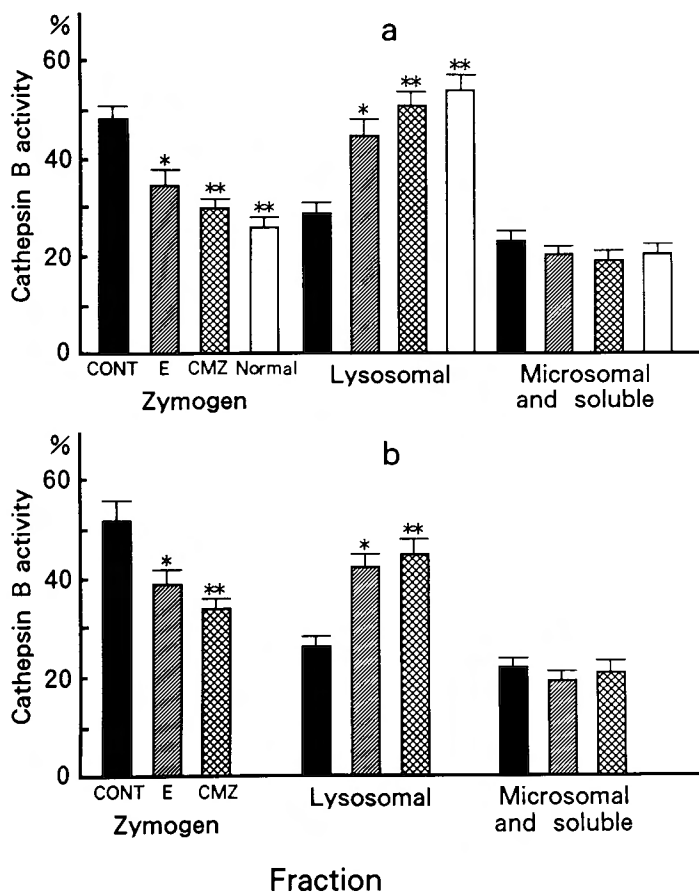


Fig. 4 Effects of E-3123 and CMZ on cathepsin B distribution in subcellular fractions of pancreatic acinar cells of mice with CDE diet-induced pancreatitis 24 (a) and 48 hours (b) after completion of CDE diet. *, $p < 0.05$; **, $p < 0.02$ compared with CONT group. Mean±SEM.

Table 3 Effects of E-3123 and CMZ on the changes in lysosomal fragility in in-vitro incubation

Group		n	Cathepsin B leakage from lysosomes (% of total) Incubation time (min)		
			30	60	90
CONT	24 hours	6	9±2	25±2 ⁺⁺	41±3 ⁺⁺⁺
	48 hours	6	10±2	28±2 ⁺	46±3 ⁺⁺⁺
E	24 hours	7	8±1	17±2 [*]	30±2 ^{***}
	48 hours	7	8±2	20±2 ⁺⁺	33±3 ^{****}
CMZ	24 hours	8	7±1	16±2 ^{**}	28±2 ^{***}
	48 hours	8	8±2	18±2 ^{**}	31±3 ^{****}
Normal mice		5	6±1	13±2	23±2

Cathepsin B leakage (soluble cathepsin B activity) was expressed as % of total. *, p<0.05, **, p<0.02, ***, p<0.01 compared with CONT group at the same stage, +, p<0.05, ++, p<0.01, +++, p<0.001 compared with normal mice. Mean±SEM.

Table 4 Effects of E-3123 and CMZ on the changes in mitochondrial fragility

Group		n	MDH leakage from mitochondrias (% of total) Incubation time (min)		
			30	60	90
CONT	24 hours	6	9±2	26±2 ⁺	44±3 ⁺⁺⁺
	48 hours	6	11±2	29±2 ⁺⁺	48±3 ⁺⁺⁺
E	24 hours	7	8±1	20±2 [*]	33±2 ^{**}
	48 hours	7	10±2	23±2 [*]	36±3 ^{**}
CMZ	24 hours	8	8±1	9±2 [*]	31±2 ^{***}
	48 hours	8	9±2	22±2 [*]	35±2 ^{***}
Normal mice		5	7±1	18±2	29±2

MDH leakage (soluble MDH activity) was expressed as % of total. *, p<0.05, **, p<0.02, ***, p<0.01 compared with CONT group at the same stage, +, p<0.05, ++, p<0.02, +++, p<0.01 compared with normal mice at the same incubation time, Mean±SEM.

against this increased lysosomal fragility, and the combination of E-3123 and CMZ was even more protective (Table 3).

The CDE diet also caused ruptures of mitochondrial enzyme (MDH)-containing organelles in vitro particularly when the incubation time was prolonged (≥ 60 min); these changes reflect the increased mitochondrial fragility induced by the CDE diet. E-3123 reduced significantly the increased fragility of mitochondria, and the combination of E-3123 and CMZ provided even more protection (Table 4).

Discussion

In young female mice, the CDE diet has been reported to cause severe acute pancreatitis with a

high mortality rate¹⁰. In this study, the CDE diet caused a high mortality rate and marked histological changes in the pancreas such as acinar cell vacuolization, interstitial edema, inflammatory cell infiltration, and tissue necrosis with high amylase levels in the serum and ascitic fluid. Moreover, the CDE diet caused marked fragility of zymogen granules, lysosomes and mitochondria and a marked shift of the lysosomal enzyme, cathepsin B, from the lysosomal fraction to the heavier zymogen fraction, indicating that lysosomal enzyme redistribution is induced by the CDE diet. This redistribution of lysosomal enzyme means colocalization of lysosomal enzyme with digestive enzymes, which has been reported to occur in two other experimental models of pancreatitis: caerulein-induced¹⁸ and pancreatic duct obstruction^{16, 19}. This colocalization phenomenon seems to be important in the pathogenesis of acute pancreatitis, because cathepsin B as a lysosomal enzyme can activate trypsinogen^{5, 17}, and trypsin can activate many other digestive enzymes. Furthermore, in this study the CDE diet caused congestion of both digestive enzyme and lysosomal enzyme in acinar cells, and this higher concentration and colocalization of lysosomal enzyme and digestive enzyme is probably dangerous since it can lead to the intracellular activation of digestive enzymes. In addition, the CDE diet caused a great increase in plasma endotoxin levels. The endotoxemia and the septic state induced by the CDE diet, probably through pancreatic infectious, suggests that infectious factors play an important role in the development of this severe pancreatitis. A new synthetic protease inhibitor E-3123, is a guanidino acid esters which has been reported to inhibit several key enzymes in pancreatitis, such as trypsin, phospholipase A₂ and elastase⁶. There have also been several reports on the protective effects of this type of new potent protease inhibitor in experimental pancreatitis^{9, 23}. Its relatively small molecular weight (508 daltons) suggests that it can penetrate into pancreatic acinar cells, and its strong inhibiting ability encouraged us to study its protective effects against the pancreatic injuries induced by the CDE diet. The administration of E-3123 alone had a significant protective effect against the increase in the mortality rate, serum amylase level, ascitic fluid amylase level, plasma endotoxin level, pancreatic amylase and cathepsin B levels and lysosomal and mitochondrial fragility. In addition, E-3123 therapy alone significantly improved the redistribution of lysosomal enzyme, but it did not significantly improve the histological changes induced by the CDE diet. The combination of E-3123 and the broad spectrum antibiotic cefmetazole had even more potent protective effects against these pancreatic acinar cell injuries induced by the CDE diet. The combination of E-3123 and CMZ improved significantly the histological changes and reduced the mortality rates more than did E-3123 alone. The combination of E-3123 and CMZ was also more effective in treating the endotoxemia induced by the CDE diet.

These results suggest that both some proteases which can be inhibited by E-3123 and an infections factor which can be treated by CMZ play important roles in the pathogenesis and development of severe pancreatitis. This new type of potent protease inhibitor in combination with a strong antibiotic may prove to be useful clinically in the treatment of severe acute pancreatitis in as early a stage as possible.

Acknowledgments

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和文抄録

マウス食餌性膵炎時での新しいプロテアーゼインヒビター E-3123 と抗生剤セフメタゾールの 併用療法による生存率の改善

京都大学 医学部 第一外科学教室

平野 鉄也, 真辺 忠夫, 大塩 学而, 仁尾 義則

急性膵炎での感染性因子の役割を検討する目的にて, マウスの CDE 食餌誘起性膵炎において, 新しいプロテアーゼインヒビターである E-3123 と広域抗菌作用を持つ抗生剤であるセフメタゾールの併用療法を検討した. この併用療法は, CDE 食餌誘起性膵炎における高死亡率, 高アミラーゼ血症, 高腹水中アミラーゼ値, 膵アミラーゼおよびライソゾーム含有量の上昇, 高エンドトキシン血症, ライソゾーム分画よりチモー

ゲン分画へのライソゾーム酵素の再分布, ライソゾームやミトコンドリア脆弱性の亢進, 膵組織学変化のすべてにおいて, E-3123 の単独療法より有意な抑制効果を示した. これらの結果は, 重症膵炎の進展において感染性因子が重要な役割を果たしていることを示唆させるとともに, 抗生剤とプロテアーゼインヒビターの併用療法が臨床の膵炎治療においても有用であることも示唆させた.