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Experimental acute pancreatitis in dog

1) Effect of urinary trypsin inhibitor (UTI) on survival rate, pathogenesis, serum amylase and TAMe hydrolytic activities

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Key words: Acute pancreatitis; amylase activity; TAMe hydrolytic activity; urinary trypsin inhibitor

SUMMARY

The effect of human urinary trypsin inhibitor (UTI) on acute hemorrhagic pancreatitis induced experimentally by the duodenal blind loop method was investigated in mongrel and beagle dogs.

Over 50% and 90% of dogs in the control group died 18 and 36 hr after preparation of duodenal blind loop, respectively. The survival rate of the UTI groups (20,000 and 60,000 U/kg, i.v.) was 50% and 80% ($p < 0.001$), respectively, demonstrating a significant inhibitory effect on the onset of pancreatitis. The direct injection of UTI into the duodenal bling loop exerted especially a potent inhibitory effect.

Histopathological examinations revealed retention of hemorrhagic ascites in all dogs of the control group, as well as extensive parenchymatous lysis and necrosis centralizing to the pancreatic lobules, its circumferential hemorrhage and infiltration of neutrophils, and lytic swelling of acinus. Neither hemorrhagic nor necrotic finding was detected in the pancreas of the UTI groups.

Serum amylase and tosyl-L-arginine methylester (TAMe) hydrolytic activities were measured during pancreatitic course. In the control group both activities increased as the time proceeded after preparation of duodenal blind loop, and 36 hr later they reached levels of over 4 times as high as the preoperative level. In the UTI groups (6,000－60,000 U/kg, i. v.) the serum amylase activity remained almost unchanged while the increase in TAMe hydrolytic activity was inhibited significantly.

The antipancreatitic effect of UTI appeared to be equivalent to or more potent than that of a conventional drug, aprotinin.

INTRODUCTION

Urinary trypsin inhibitor (UTI) is one of the serial inhibitors stable to and soluble in acids[1-11]. These compounds are currently designated as acid-stable trypsin inhibitor (ASTI). UTI is known to have a broad

inhibitory spectrum against various enzyme activities obtainable from human urine[4-12]. We have studied the change in UTI activity under various pathologic conditions[13-15] and properties mainly from the molecular structural stand-point[5,8,10,12,16-20]. As the results, we have found: (1) that the naturally occurring UTI is a glycoprotein of a single chain structure having alanine (Ala) at the N-terminal, with molecular weight of approximately 70,000 by gel filtration method[11,19,20] (2) that UTI of this type is degraded to lower molecules by the actions of various serine- and SH-proteases[21-24], and (3) that its inhibitory activity along with molecular forms is altered by chemical modification[25-27]. On a basis of a considerable literature documenting that UTI shows a potent inhibitory activity on trypsin and chymotrypsin[4-12,16,17], we demonstrated first in 1978 that the survival rate of dogs with experimental acute pancreatitis (induced by the Elliott's method) could be increased by UTI and also the drug inhibited activation of blood enzymes such as lipase and amylase even though there were some differences in inhibitory activities[28]. The histopathological picture of this pancreatitic model differs slightly from that of human fulminant pancreatitis[29]. However, the antipancreatitic effect of UTI was confirmed by Onishi et al.[30] in the same experimental model dogs a few years later. The urinary excretion rate of UTI is closely correlated with that of steroid hormones. It has been also verified: (1) that UTI is produced enzymatically from a kind of precursor (pro-inhibitor) when serine- and SH-proteases in blood are activated[20,21,33,34], and (2) that ASTI which has the same antigenicity as UTI is widely distributed in human body fluid[38-43] or tissues[44-46] when determined by micro-assay utilizing antigenicity such as the recently developed enzyme-linked immunosorbent assay[35-37]. Anti-shock[47], anti-ulcer[48] and immunosuppressive[48] (in skin grafting etc.) effects of UTI have gradually been clarified by the studies of different administration routes. Furthermore, it is presumed currently that UTI or related inhibitors play a role in not only controlling specific blood enzyme systems but also acting as an important bioactive factor most closely relevant to vital phenomenon. To elucidate the effect of UTI more in details, in this study its purified natural form was administered to dogs with experimental acute pancreatitis induced by the method of Pfeffer et al.[49] which has been accepted as most resembling human acute pancreatitis[29].

MATERIALS and METHODS

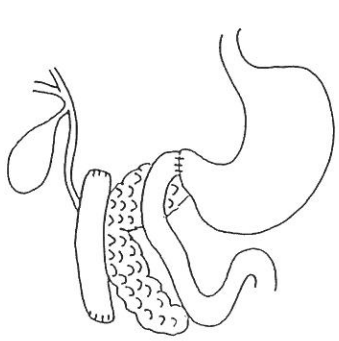
1. *Drug*

UTI generously supplied by JCR(Kobe, Japan)and used in this study was highly purified preparation (Lot 007E) derived from human fresh urine. Its molecular weight and specific activity were approximately

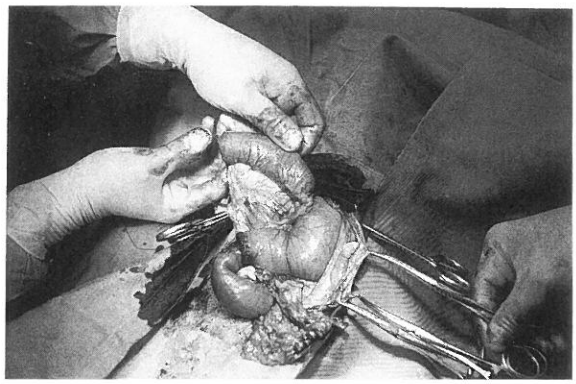
67,000(gel filtration)and 2,700 U/mg protein without pyrogen, respectively. Aprotinin and tosyl-L-arginine methylester (TAMe) were purchased from Bayer (West Germany) and Peptide Research Laboratory (Osaka, Japan), respectively.

2. *Animals*

Adult mongrel dogs (36 males, 2 females) and adult beagle dogs (10 males, 2 females), weighing about 10 kg and previously confirmed to be free from abnormality in coagulation-fibrinolysis system, were fasted for 24 hr before use.



a)Schematic presentation for operation



b)Gross finding immediately after operation

Fig. 1. Duodenal blind loop method(Pfeffer's modification)

3. *Preparation of experimental pancreatitis*

Each dog was laparotomized by midline incision under pentobarbital Na anesthesia (Abott, 25 mg/kg body weight, i.v.), and experimental pancreatitis was induced by the method of Pfeffer et al.[49]. The stomach was separated from the duodenum by cutting at the pyloric part, and duodenal blind loop of 7 - 11 cm length including pancreatic duct and accessory pancreatic duct was prepared. Then, the end-to-end anastomosis of the cut stomach and duodenum was performed for reconstruction of the digestive tract (Fig. 1). Separately, dogs were laparotomized by median incision under pentobarbital Na anesthesia (i.v.), the abdomen was closed by the Czerny-Lembert's suture without abdominal procedure (sham operation), and served as the control group. All surgical procedures described above were carried out under sterile condition as far as possible. A 18G-polyethylene cannula was indwelled in the incised vein about 15 cm toward the CNS side from right great saphenous vein, and blood was drawn in time course.

Animals were divided, according to kinds of drugs and dosage as well as the method of injection, into 6 groups as follows.

(a)Each drug was continuously injected i.v. for 4 − 5 hr, starting immediately after preparation of the duodenal blind loop: UTI 3, 000 U/kg (dogs A), UTI 10, 000 U/kg (dogs B), UTI 30, 000 U/kg (dogs C), and aprotinin 60, 000 KIU/kg (dogs D). UTI or aprotinin was also injected i.v. to the above dogs immediately before, immediately, 6 and 12 hr after preparation of the duodenal blind loop: UTI 750 U/kg each to dogs A totalling 6, 000 U/kg (group A), UTI 2, 500 U/kg each to dogs B totalling 20, 000 U/kg (group B), UTI 7, 500 U/kg each to dogs C totalling 60, 000 U/kg (group C), and aprotinin 15, 000 KIU/kg each to dogs D totalling 120, 000 KIU/kg (group D).

(b)In some dogs, UTI was injected directly into the duodenal blind loop by taking for 4 − 5 continuous hr immediately after preparation of the duodenal blind loop: UTI 3, 000 U/kg (dogs E) and UTI 10, 000 U/kg (dogs F). In addition, UTI was injected i.v. to these dogs immediately before, immediately, 6 and 12 hr after preparation of the duodenal blind loop: UTI 750 U/kg each to dogs E totalling 6, 000 U/kg (group E) and UTI 2, 500 U/kg each to dogs F totalling 20, 000 U/kg (group F).

4. *Apothanasia effect*

The apothanasia effect of UTI was determined in terms of survival rate of dogs 18, 24 and 36 hr after induction of the experimental pancreatitis.

5. *Histopathological examination*

Dogs surviving still 36 hr after preparation of the duodenal blind loop were sacrificed by the additional i.v. injection of excessive pentobarbital Na. Postmortem examination was performed immediately after sacrifice or immediately after death owing to any causes other than sacrifice. Along with gross examination, the specimen of pancreatic tissue with grossly suspected critical damage was fixed in 10% formalin followed by hematoxylin−eosin staining, and the microscopic observation was proceeded.

6. *Measurement of serum amylase*

Venous blood samples were collected by the double step method before operation and 0, 3, 6, 12, 18, 24 and 36 hr after preparation of the duodenal blind loop. Amylase activities in separated sera were measured by the saccharogenic method[50].

7. *Measurement of TAMe hydrolytic activity*

Plasma tryptic enzyme activity was measured by the Hestrin's method[52] modified by Roberts[51] in the presence of a synthetic substrate, 15 mM TAMe adjusted by 0.1M phosphate buffer of pH 7.4.

RESULTS

1. *Survival rate of dogs after induction of acute pancreatitis*

Of 10 mongrel and beagle dogs (control group), 6 and 3 totalling 9 died 18 and 36 hr after preparation of the duodenal blind loop induced by the Pfeffer's modification method, respectively (Fig. 2 a). In the group A(6,000 U/kg), almost no apothanasia effect of UTI was observed. The survival rates in the groups B(20,000 U/kg) and C(60,000 U/kg) 36 hr after preparation of the duodenal blind loop were 50% and 80% ($p<0.001$), respectively, indicating a significant apothanasia effect. Especially in beagle dogs (Fig. 2b), the survival rate in the control group was 0% at and after 24 hr of the operation whereas that in the group C was 80% which was indicative of a potent apothanasia effect of UTI. The survival rate in the group D (aprotinin 120,000 KIU/kg) at 36 hr after the operation was 60% ($p<0.05$, Fig. 2a). The survival rate in the group F(20,000 U/kg, Fig. 2a) was 100% even though the number of animals used was small, i.e., UTI injected directly into the duodenal blind loop was proved to show an excellent apothanasia effect.

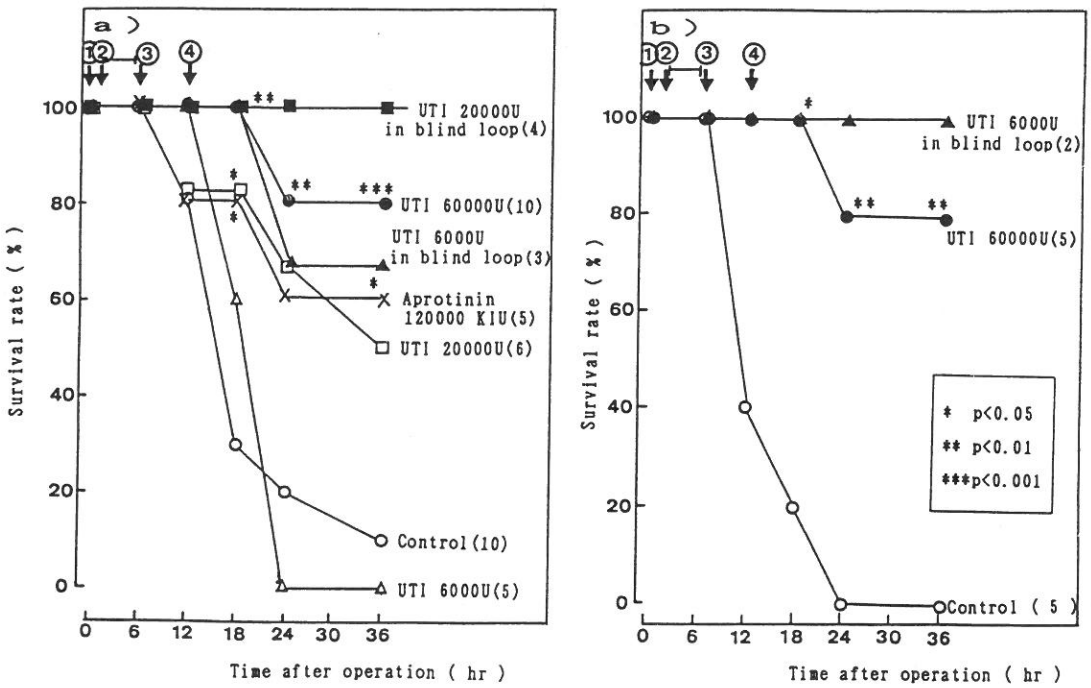


Fig. 2. Effect of UTI on survival rate of dogs with acute pancreatitis
a) All dogs (Mongrel and beagle dogs totalling 50 heads)
b) Beagle dogs (Total: 12 heads)
Thick solid horizontal line and ① – ④ present the time for continuous i.v. injection and usual i.v. injection of each drug, respectively. Figures in parentheses represent number of dogs used for the experiment.

2. *Gross and histopathological examinations of pancreas*

The postmortem examination was performed on dead and sacrificed dogs. As the result, a moderate amount of retention of hemorrhagic ascites and necrosis of the blind loop with dilatation were detected in all dogs of the control group. Particularly, the hemorrhagic and necrotic changes in pancreas were observed extensively and markedly, and abnormalities from the head to the body of pancreas were seen in remarkably altered cases (Fig. 3a). The histopathological findings shown in Fig. 4a were extensive parenchymatous lysis and necrosis centralizing to the lobules and its perilobular hemorrhage and infiltration of neutrophils as well as lytic swelling of acinus. Contrarily, the dogs of the UTI treated groups were characterized by expansion of the duodenal blind loop, hemorrhage of the wall, pulmonary edema and inflammation of pancreas without hemorrhagic and necrotic picture of pancreas. These findings verify and efficacy of UTI (Fig. 3b, Fig. 4b). No inflammatory change was recognized around and in the pancreas of the sham operated group.

3. *Action on blood enzymes*

Fig. 5a shows serum amylase activities in mongrel dogs with experimental acute pancreatitis. Serum amylase activities increased with the progress of time after preparation of the duodenal blind loop; the activities 12 and 18 hr later became 3 times ($2,695 \pm 937$ U) and about 4 times ($3,362 \pm 400$ U) higher than the preoperative value ($1,287 \pm 312$ U), respectively. The increases in both values were statistically significant ($p<0.005$ in both cases).

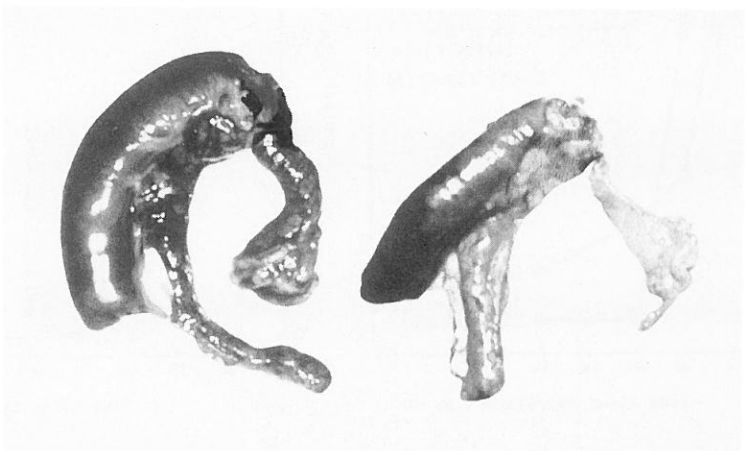


Fig. 3. Gross findings on the pancreas immediately after operation and on pancreatitis
a)36 hr after operation(Control)
b)UTI 60,000 U/kg, i.v.
Hemorrhagic necrosis is seen in the pancreas of the control group with acute pancreatitis.

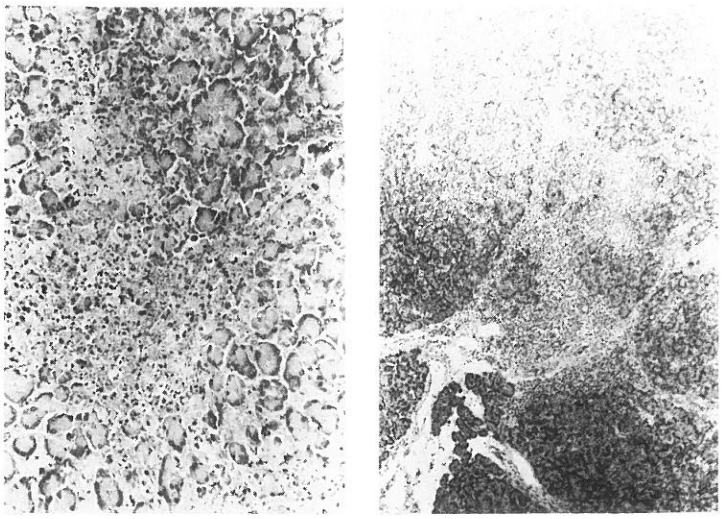
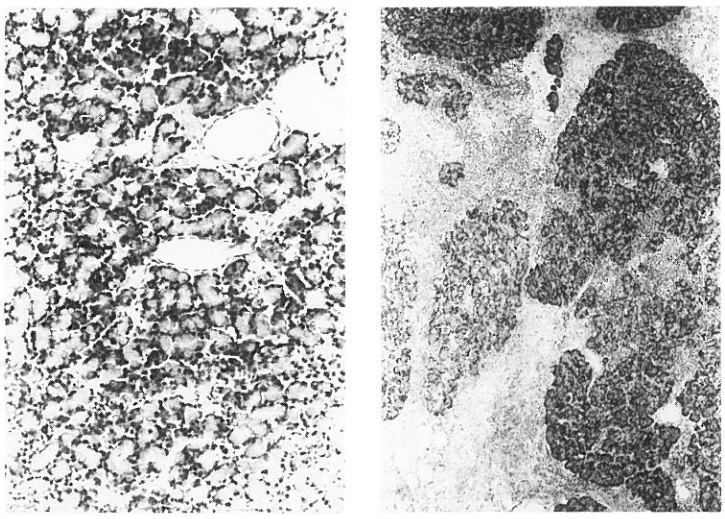


Fig. 4. Microscopic photo of pancreatic tissue(H-E staining, x100 and x50)
a)36 hr after operation(Control)



b)UTI 60,000 U/kg, i.v.
Infiltration of neutrophils accompanied with marked parenchymatous histolysis and hemorrhage are observed in the control group with acute pancreatitis.

Fig. 5c shows serum amylase activities in beagle dogs with acute pancreatitis. The serum amylase activities 12 and 18 hr after the operation were about 2,558±792 U and 3,805 U, respectively, which were almost in agreement with the values in mongrel dogs.

The serum amylase activity tended to be inhibited in the UTI groups (6,000-60,000 U/kg), The inhibitory effect being weak without significant difference from the control group ($p>0.1$).

Fig. 6a shows plasma TAMe hydrolytic activity in mongrel dogs with acute pancreatitis. The change in activity up to 6 hr later (1.70 ±

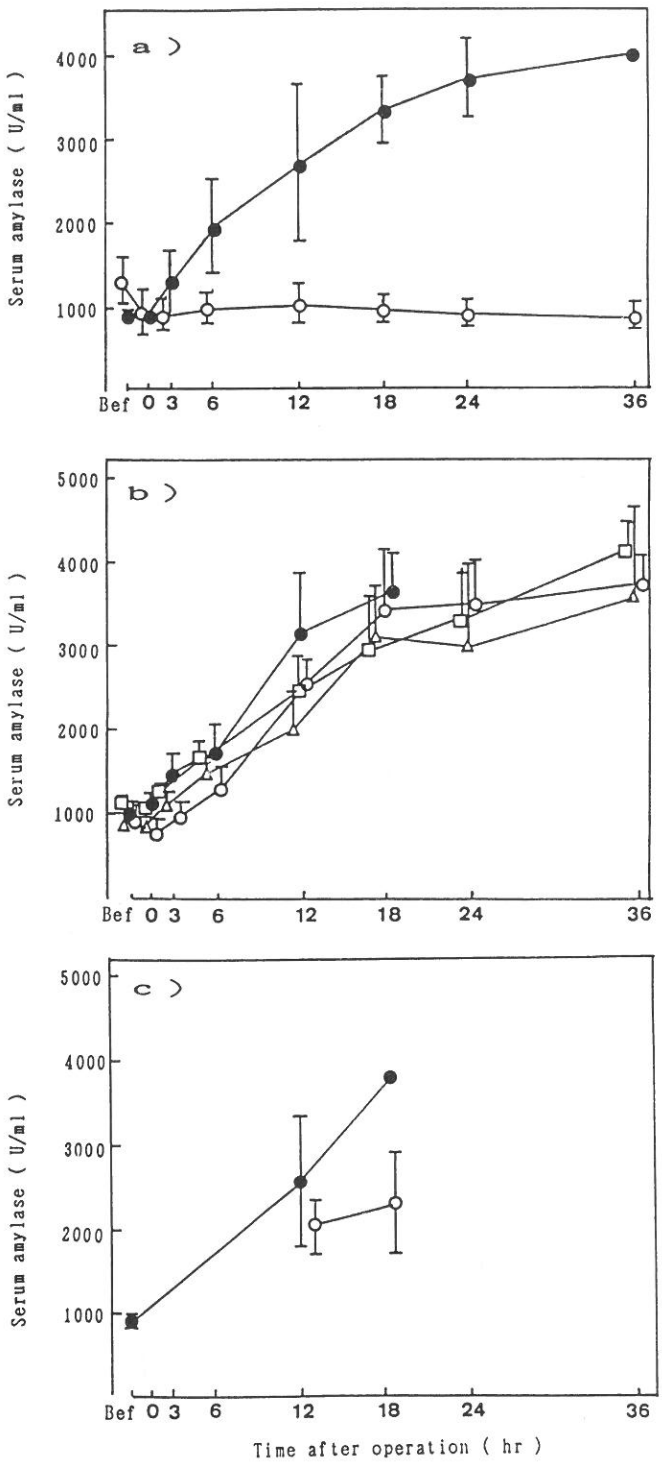


Fig. 5. Time course change in serum amylase activity
a) Mongrel dog control groups (●——● Acute pancreatitis, N= 5 ; ○——○ Sham operation, N= 5)

b) Mongrel dog UTI groups (△——△ UTI 60,000 U/kg, N= 5 ; ○——○ UTI 20,000 U/kg, N= 6 ; ●——● UTI 6,000 U/kg, N = 5 ; □——□ Aprotinin 120,000 KIU/kg, N= 5)

c) Beagle dog control and UTI groups (●——● Acute pancreatitis, N= 5 , ○——○ UTI 60,000 U/kg, N= 5)

0.74 μmole) was not so remarkable; the activity was about 3.4 times ($p<0.01$) as high as the preoperative activity (0.50±0.27 μmole). However, thereafter the activity increased rapidly to become about 12 times (6.19 ±1.95 μmole) and about 18 times (8.78±3.02 μmole) higher than the preoperative value 12 and 18 hr after induction of experimental acute pancreatitis, respectively ($p<0.001$ in both cases). In the case of beagle dogs (Fig. 6 b), the result was similar to that in mongrel dogs; at the same measuring points the activities (about 5.11 and 9.00 μmole, respectively) were nearly equal to those in mongrel dogs. Unlike the serum amylase activity, these plasma TAMe hydrolytic activities were inhibited quite strongly by UTI.

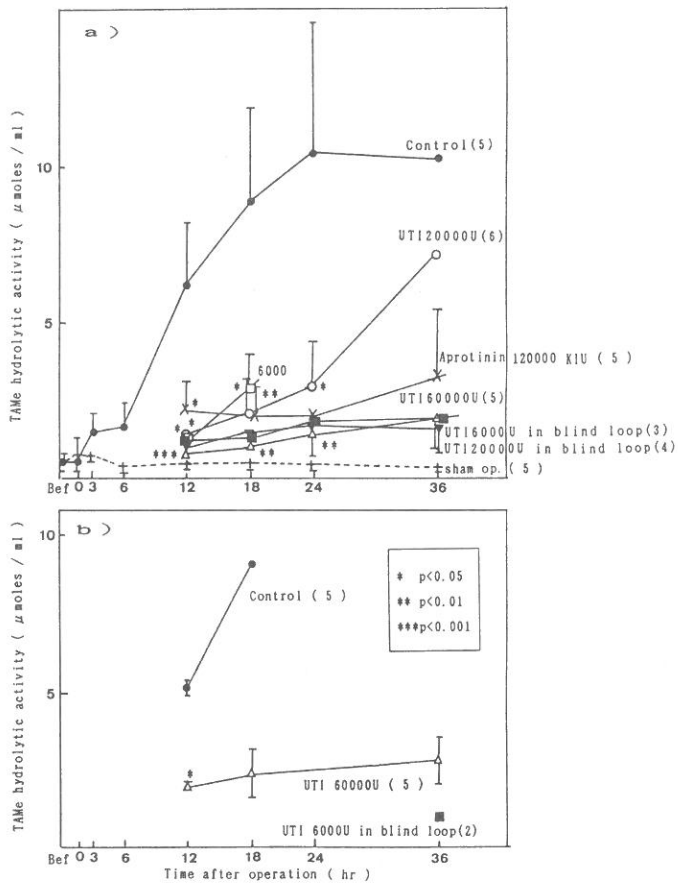


Fig. 6. Time course change in blood protease activity
a) Mongrel dogs(Total: 38 heads)
b) Beagle dogs(Total: 12 heads)
Figures in parentheses denote number of dogs used for the experiment.

Fig. 7 summarizes the effect of aprotinin used for comparison with that of UTI in all pancreatitic animals (45 dogs). The activities 12 hr (by UTI of a relatively small dose, 6,000 U/kg) and 18 hr (by UTI at over 20,000 U/kg) after preparation of the duodenal blind loop were

significantly lower than that of the control group, which were almost similar to the preoperative value. Thus, UTI was found to have a signif-icantly potent inhibitory effect ($p<0.001$) on plasma TAMe hydrolytic enzymes. Although the number of dogs examined were small, it was found that the direct injection of UTI into the duodenal blind loop could inhibit significantly the plasma TAMe hydrolytic activity and its inhbitory effect was never inferior to that of aprotinin of 120,000 KIU/kg.

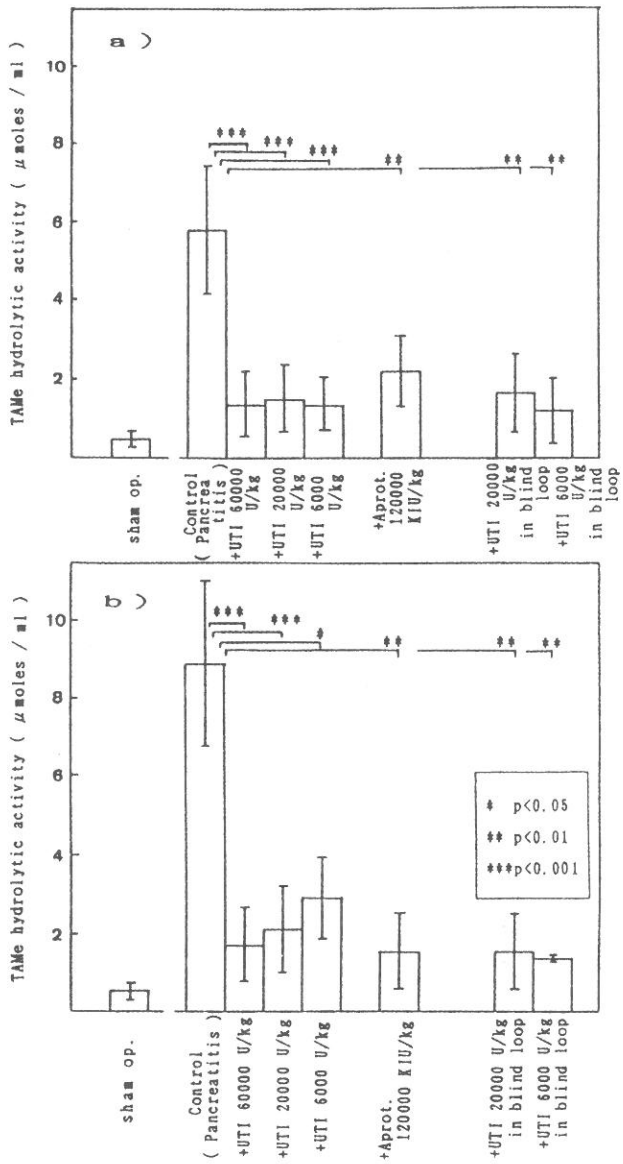


Fig. 7. Effect of UTI on TAMe hydrolytic enzyme
a)Effect of UTI on all dogs(total:50 heads)12hr after operation to induce acute pancreatitis
b)Effect of UTI on all dogs 18 hr after operation

DISCUSSION

UTI was found to have a potent apothanasia efficacy in dogs with acute pancreatitis prepared by the duodenum ligating loop method which is accepted for preparing a model of most resembling postoperative acute pancreatitis in man. While 90% of dogs in the control group died 36 hr after the operation, more than 80% of dogs ($p<0.001$) could survive in the group C (60,000 U/kg): an excellent apothanasia effect of UTI could be confirmed. These survival rates surpassed the results reported by us[28] and Onishi et al.[30] in dogs with pancreatitis induced by Elliott's method.

Activation of pancreatic trypsin had generally been regarded as an important trigger of acute pancreatitis[53-55]. Based on this view, pancreatitis had been induced trially by the injection of trypsin or trypsin-bile acid into the pancreatic duct as the method by Elliott et al.[56]. Active trypsin acts on not autolysis[57] but kininogen to release bradykinin and simultaneously to activate plasma kallikrein[58]. When active trypsin escapes into blood, it causes direct hemolysis, topical vasoconstriction and stimulation of posterior peritoneum nerve to result in shock and also activates elastase, phospholipase A_2 and other enzymes[55,59]. The activated enzymes decompose secondarily elastin of vascular wall to elicit hemorrhage or act on lecithin to form lysolecithin which provokes damage of cell membrane, resulting in multiple organ failure (MOF) in prognosis of acute pancreatitis. Therefore, it is imperative to inhibit first the primary trigger enzyme activity for preventive purpose of pathogenesis of pancreatitis. In this sense, UTI is an ideal drug having a potent anti-trypsin activity ($Ki=10^{-11}$ M order)[5,6,8,26]. UTI also acts on pancreas-derived chymotrypsin, kallikrein and elastase[12,60] and inhibits leukocytic elastase[19] being a strong cellular protease, cathepsin G and acrosin. These actions are not found in aprotinin. From these actions, UTI is presumed to exert broad inhibitory effects on various enzymes to be produced secondarily by pancreatic trigger. In this study, it was of great interest to know that plasma TAMe hydrolytic activity was inhibited strongly by UTI almost in agreement with the apothanacia effect of UTI and that the inhibitory effect was nearly concentration dependent, e.g., in the group C (the highest concentration, 60,000 U/kg) the inhibitory effect of UTI lasted over long periods of 12 – 18 hr. In our previous communications[62-64] it was shown that kallikrein, trypsin or elastase which were released from the pancreas of patients with acute pancreatitis reacted mainly with blood α_2-macroglobulin and that the captured enzyme activities were hardly measured in high molecular substrates such as caseine or hemoglobin, but the activities could be measured in a low molecular substrate, TAMe. TAMe can also serve as the substrate for not only trypsin but also enzymes of blood kallikrein-kinin system, coagulation-fibrinolysis system or complement system[54,55]

which are all considered to be closely related with the onset of pancreatitis. That this TAMe hydrolytic activity is almost completely inhibited by UTI of 20,000 U/kg or higher concentrations (Fig. 7a, 7b) or of 6,000 U/kg of lower dosage up to a 12-hr postoperative period (Fig. 7-a) suggests a potent inhibitory effect of UTI on various enzyme systems in vivo. However, the effect of UTI on amylase which is activated by pancreatitis was weak. In postoperative course, the increase in amylase activity is frequently derived from glandular organs particularly other than pancreas[65]. On the contrary, serum amylase activity is often reported to become low in critical cases such as lethal pancreatitis[65]. Therefore, serum amylase activity is not always a good index for the pathologic condition of pancreatitis. Further studies on the relationship between UTI and these enzymes in various substrates are necessary.

Although animals used were few, the direct injection of UTI into the duodenal blind loop inhibited pancreatitis stronger than its i.v. injection. Opie[67] and Quick[68] once proposed a duodenal regurgitation theory as the pathogenesis of acute pancreatitis. When we take this theory into consideration, the injection of UTI into the duodenal blind loop is considered as a most rational therapeutic method because UTI acts directly on the trigger enzymes of pancreatitis and enzymes to be produced secondarily. Further studies into the method of administration of UTI is believed to bring about a clinically useful prevention against acute pancreatitis.

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