Role of Endogenous Cholecystokinin in the Regeneration of Pancreatic Tissue after Acute Hemorrhagic Pancreatitis in Rats

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Abstract  The aim of this study was to investigate the effect of endogenous cholecystokinin (CCK) on pancreatic regeneration after acute hemorrhagic pancreatitis. Acute hemorrhagic pancreatitis was induced in rats by two intraperitoneal cerulein injection (20 μg/kg BW) with 5h water-immersion stress once a day for successive 3 days. After the cessation of repetition of acute pancreatitis the rats were treated with successive feeding with 0.1% camostat-containing diet or SC injection of CR-1505 (CCK receptor antagonist, 50 mg/kg BW × 2/day) for 7 days. Zymogen enzymes and protein contents per DNA in pancreatic tissue were significantly higher in rats treated with camostat compared with control rats, and plasma CCK level was elevated. To the contrary, pancreatic regeneration was retarded in the rats treated with CR-1505. It is concluded that endogenous CCK has a trophic effect during regeneration after acute hemorrhagic pancreatitis.

Key wards: stress-cerulein induced pancreatitis, cholecystokinin, camostat, CR-1505, regeneration,

Introduction

It is now well documented that pancreatic growth is controlled by several factors, including cholecystokinin (CCK), secretin, gastrin, insulin, neurotensin, and epidermal growth factor. Among them, CCK and its analog seem to be the most potent growth factor and have been most widely studied. All these studies, to prove the trophic affect of CCK on pancreas, involved either of 4 respects mentioned below: 1. feeding protease inhibitors or a high-protein diet. 2. surgical procedures such as pancreatico-biliary diversion, jejunal bypass with infusion of nutrient solution into jejunum or 60% distal resection of pancreas. These two accelerated endogenous CCK release. 3. administration of exogenous CCK or its analog, cerulein or proglumide. 4. administration of CCK receptor antagonists (L-364, 718, CR-1409, CR-1505, MK-329 or proglumide) to interfere the combination of CCK with its receptor. Most of these studies investigated the effect of CCK on normal pancreas growth, and the reports studying the regenerative affect of CCK on damaged pancreas are few. Up to now, no studies have investigated the correlative changes of plasma CCK concentration with the time course of acute hemorrhagic pancreatitis and subsequent regeneration.

The aim of this study was to investigate the effect of endogenous CCK on the regeneration
of pancreatic damage in rats, which was induced by water-immersion stress and excessive dose of cerulein, and at the same time to determine the corresponding changes of CCK level in plasma.

Materials and Methods

Materials
Cerulein (Ceosumin®) was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Camostat and CR-1505 were presented by Ono Pharmaceutical Co., Ltd., Osaka, Japan and Tokyo Tanabe Co., Ltd., Tokyo, Japan, respectively. Camostat contained feed was ordered from Oriental Yeast Co., Ltd. Tokyo, Japan. Benzoylarginine ethyl ester hydrochloride (BAEE) was purchased from Nakarai Chemicals, Ltd., Kyoto, Japan. Succinyl-L-alanyl-L-alanyln-p-nitroanilide (Suc-(Ala)₂-NA) was purchased from Peptide Institute, Inc., Osaka, Japan. Purified porcine enterokinase and bovine trypsin were purchased from Sigma Chemical Co., St. Louis, MO, USA. DNA from salmon spermary was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Animals
Male Sprague-Dawley rats weighing 350-450g were fed a standard laboratory diet and water ad libitum, and kept in cages in a temperature (22±2°C) and humidity-controlled (55±5%) room with a 12h dark-light cycle (7PM-7AM dark cycle) before and during the experiment. The rats were maintained throughout the study according to guidelines of the committee on animal care of Kyushu University.

Experimental Procedure
Twenty-two rats were randomly divided into 4 groups. Acute hemorrhagic pancreatitis was induced in all rats as follows. 20 μg/kg body weight of cerulein was injected intrapelitonally twice daily at 9AM and 10AM, and rats were placed in individual close-fitting restraint cages, and immersed in water (22-23°C) for 5 hours (9AM-2PM). This pancreatitis was induced repeatedly for 3 days. On 4th day rats (n=5) of control group were killed after an overnight fast, and pancreas was removed. From 4th day other rats of each group were treated differently. In Camostat group, rats (n=5) were given 0.1% camostat contained feed instead of the standard laboratory diet ad libitum for 7 days. In CR group, rats (n=5) were injected 2% CR-1505 saline solution SC (50 mg/kg BW) twice a day (at 9AM and 5PM) and fed with standard laboratory diet for 7 days. Remaining rats (n=5) of control group were killed at 9AM everyday. On 12th day, after an overnight fasting, the rats were anesthetized with an IP pentobarbital injection (40 mg/kg BW) to collect peripheral blood from the inferior vena cava for assaying the CCK content in plasma. Then the rats were killed by decapitation and the pancreata were quickly removed, freed from fat and lymph nodes on ice, and pancreatic wet weights were weighed. Four rats without receiving pancreatitis were killed as a normal control.

The pancreatic tissue was divided into 3 parts. One part of the pancreas was fixed immediately in 20% formalin and stained with H & E for histological assessment. Another part was stored at −80°C until measurement of DNA content in pancreas tissue. The remaining part of the pancreas was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100, and the supernatant was obtained as previously described¹¹ for the measurement of the enzymes content in pancreatic tissue. No rats died during the experiment with these procedures.

Assay
Amylase content in pancreas tissue was measured by the method of Ceska et al.⁹ using Phadebas amylase test (Pharmacia Laboratories, Piscataway, N. J. USA). Trypsin content in pancreatic tissue was measured en-
zymatically with BAEE as a substrate after the activation of trypsinogen with enterokinase as previously reported. Elastase in pancreatic tissue was measured enzymatically with Suc-(Ala)_{3}-NA as substrate after the activation of proelastase with trypsin. Each enzyme content was expressed as units of enzyme activity per microgram DNA. Pancreatic tissue DNA and protein were measured according to the methods of Burton and Lowry, respectively.

Rat plasma CCK the 12th day after the first cerulein injection was measured using OAL-656 antibody and ¹²⁵I-CCK as a standard. CCK-8 (sulfated) was used as a standard. The CCK values were expressed in fmol/ml. CCK fraction was extracted from 0.5 ml of plasma using Sep-Pak C18 and was reconstituted to 0.5 ml with assay buffer [30 mM sodium phosphate buffer (pH 7.6), 0.1% BSA, 0.02% sodium azide and 200 KIU/ml trasyrol]. 0.2 ml of extract was subjected to radioimmunoassay and all the assays were duplicated.

Statistical Analysis

All results were expressed as mean±SEM. The statistical significance of differences between control, Camostat, and CR group on 12th days was evaluated with ANOVA one-way analysis of variance. The two-tailed Student's unpaired t-test was used for the statistical analysis for the normal group and control group (4th and 12th day).

Table 1  Changes of Body Weight after the Repetition of Acute Pancreatitis

<table>
<thead>
<tr>
<th></th>
<th>0day</th>
<th>4th day</th>
<th>11th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (5)</td>
<td>100</td>
<td>93.2±0.7</td>
<td>99.0±0.9</td>
</tr>
<tr>
<td>Camostat group (6)</td>
<td>100</td>
<td>91.2±1.6</td>
<td>95.8±1.8</td>
</tr>
<tr>
<td>CR group (6)</td>
<td>100</td>
<td>91.9±0.4</td>
<td>102.6±0.9</td>
</tr>
</tbody>
</table>

Each value indicates percentile of the body weight compared with the body weight of the 0day. Each value means mean±SEM. Control group: fed normal diet for 7days after cessation of 3repetitions of stress-cerulein induced pancreatitis. Camostat group: fed 0.1% camostat containing diet for 7days after the cessation of repetition of acute pancreatitis. CR group: injected CR-1505 (50mg/kgBW×2/day, SC) for 7days after the cessation of repetition of acute pancreatitis. Significant difference represented *p<0.05.

Results

Change of Body Weight after Repetition of Acute Pancreatitis

After three repetitions of pancreatitis, decrease of body weight of rats was observed in all three groups. On 11th day after the first induction of the pancreatitis, the body weight in CR group recovered to the original level (0 day) earlier than other two groups. There was a significant difference in body weight between Camostat and CR groups, but not between control group and these two groups (Table 1).

Change of Pancreatic Wet Weight after Repetition of Acute Pancreatitis

Pancreatic wet weight kept decreasing after the repetition of acute pancreatitis in control group, and in CR group the decrease of pancreatic wet weight was accelerated more comparing with control group. In Camostat group pancreatic wet weight recovered to normal level on 12th day after the first induction of acute pancreatitis (Figure 1).

Change of Zymogen Enzymes Contents in Pancreatic Tissue after Repetition of Acute Pancreatitis

Although enzyme contents per DNA in pancreatic tissue decreased markedly just after the repetition of acute pancreatitis (on the 4th day), they, except for trypsin content, returned to normal levels on the 12th day in control
group. In Camostat group amylase, trypsin and elastase contents were markedly higher as compared with those of control group. In CR group amylase and elastase contents were statistically lower as compared with each value of control group (Figure 2).

**Change of Protein Content per DNA in Pancreatic Tissue after Repetition of Acute Pancreatitis**

Protein content per DNA in Camostat group was significantly higher than that of control group on the 12th day after the first induction of pancreatitis, but there was no difference in protein content between CR group and control group (Figure 3).

**Change of DNA Content in Pancreas after Repetition of Acute Pancreatitis**

DNA content per pancreas decreased to about one third of normal in control group on the 12th day. There was a significant difference in DNA content between Camostat and CR groups, but no significant differences between these two groups and the control group on the 12th day (Figure 4).

**CCK Level in Plasma after Repetition of**
**Discussion**

The present study showed that camostat accelerates the regeneration of pancreas after severe acute hemorrhagic pancreatitis, while CR-1505 prevents the recovery of pancreatic tissue, and indicated elevated plasma CCK in camostat treated group.

There are many reports investigating the trophic effect of CCK on pancreas. These reports are divided into two types regarding the style of experiment, investigating the effects of CCK on normal pancreatic growth or on regeneration after pancreatic damage. As to the effect of CCK on normal pancreas, most of the studies proved that both of hypertrophy and hyperplasia were induced on normal pancreas by elevated CCK or its analog, but a few reports said that only pancreatic hyperplasia was observed. These accelerated pancreatic growth due to elevated CCK was prevented by CCK receptor antagonists, but the effect of CCK receptor antagonist alone on normal pancreatic growth remains unclear. Some reports said that CCK receptor antagonist did not alter normal pancreatic growth, but others showed that it prevented normal pancreatic growth. Therefore it is true that elevated CCK and its
Endogenous CCK and regeneration of pancreas

Fig. 5 Microscopic findings. A: Pancreas tissue on the 4th day after the first induction of pancreatitis. Tubular complexes with normal acini are visible (×170). B: Pancreas tissue in control group on the 12th day. Regenerative acinar cells lacking polarity are observed (×170). C: Pancreas tissue in Camostat group on the 12th day. Lots of regenerated acini having rich zymogen are observed (×85). D: Pancreas tissue in CR group on the 12th day. Tubular complexes are well observed (×85).

analog promote normal pancreatic growth, but not clear whether endogenous CCK plays a role in the maintenance of normal pancreatic growth. On the other hand there were few reports concerning the effects of CCK on regeneration after pancreatic damage. Pancreatic damage was induced by DL-ethionine injection with protein-free diet, 60% distal resection of pancreas or excessive dose of cerulein.

According to previous reports cerulein alone never induce hemorrhagic pancreatitis in animals, no matter how high the dosage of cerulein is. However, cerulein induced edematous pancreatitis developed into hemorrhagic pancreatitis under water immersion stress in rats. In this study this stress-cerulein induced pancreatitis was repeated for 3 successive days. Under these conditions, rather severe pancreatic damage was induced with pronounced decrease of enzyme and DNA contents, but without significant decrease of protein content. Decrease of DNA content means loss of cells. This severe pancreatic damage was proved histologically. Acinar structure was destroyed.
and acinar cells containing zymogen granules markedly decreased, as shown in Figure 5. Tubular complexes originating from degenerated acini were well observed. In control group 8 days after the cessation of induction of pancreatitis, although pancreatic wet weight, trypsin, and DNA contents in pancreas were significantly lower as compared with each original value at the 0 day, elastase and amylase contents have recovered to the normal levels. Protein content per DNA was higher than original content at the 0 day. This indicates that spontaneous pancreatic regeneration, especially hypertrophy, had begun after the severe pancreatic damage. CCK level on the 12th day was higher than that on the 4th day in control group. Majumdar et al. demonstrated pancreatic hypertrophy after DL-ethionine induced pancreatic damage in rats\(^{21}\), while Pap et al. observed hypertrophy and hyperplasia after 60% distal resection of the pancreas in rats\(^{22}\). This difference of spontaneous regeneration pattern may depend on the difference of pancreatic damage. Partial resection of pancreas means only cell reduction and does not involve inflammation.

In the present study administration of camostat, a compound of highly potent protease inhibitor, for 7 days did not alter DNA content per pancreas as compared with control group, but increased pancreas wet weight, zymogen enzyme contents other than trypsin, and especially protein per DNA significantly. Histologically acini having abundant zymogen granules increased, and size of acini appeared larger than those in other groups. These results mean that camostat accelerated the regeneration of damaged pancreatic tissue, characterized not in hyperplasia but hypertrophy. It is well known that there exists a negative feedback mechanism in pancreatic exocrine secretion. The inhibition of protease activity in duodenum stimulates the pancreatic secretion, probably due to elevated CCK\(^8\), secretin\(^{39}\) and neurotensin\(^{40}\). Recently it is reported that CCK release is stimulated by CCK releasing peptide from duodenum, and that this CCK releasing peptide is degraded by active trypsin in duodenum in rats\(^{40}\). Protease inhibitor, camostat, inhibits this degradation of CCK releasing peptide by the inactivation of trypsin. Therefore endogenous CCK release is accelerated by retained CCK releasing peptide. Plasma CCK level in Camostat group was really higher than that in control group on 12th day. In this study we used CCK receptor antagonist, CR-1505, to abolish the effects of endogenous CCK, and CR-1505 decreased pancreatic wet weight further and suppressed the recovery of elastase and amylase contents, but there was no significant differences in protein and DNA contents compared with control group. Histologically, tubular complexes almost occupied the pancreas, and acini having zymogen granules are rare. This means that blockade of CCK binding with its receptor retards the regeneration process of pancreas. In accordance with our study, previous reports demonstrated that pancreatic regeneration process was accelerated by CCK-8\(^{21(25)}\), soy bean trypsin inhibitor\(^{14}\), cerulein\(^{10}\) or protein rich diet\(^{41}\) and retarded by CCK receptor antagonists\(^{42(25)}\). These results may imply that CCK initiates pancreatic recovery from partial damage.

On the 11th day there was a statistical difference in body weights between Camostat and CR groups. We suspect that body weight did not affect the process of regeneration of damaged pancreas, because in Camostat group regeneration of pancreas was accelerated in spite of the retardation of body weight gain.

In conclusion, endogenous CCK is released during regeneration period, and plays an important role in the initiation of pancreatic recovery from pancreatic damage.

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