Original

Effects of L-364,718 and Camostat Mesilate on Pancreatic Regeneration in Rats after Caerulein-induced Acute Pancreatitis

Atsuko Hirata, Junichi Niikawa, Taiji Kawada, Hitoshi Yoshida, Mikio Kurabayashi, Yuko Kawada, Hideki Omachi, Shigeo Shin, Shigeki Tanaka, and Keiji Mitamura

Abstract: We examined the effects of a potent cholecystokinin (CCK) receptor antagonist, L-364,718 and those of a trypsin inhibitor, camostat mesilate (camostat) after induction of acute pancreatitis with caerulein. Seven intraperitoneal caerulein injections (15 μg/kg) were repeated in rats at hourly intervals. The rats were then divided into four treatment groups: oral administration of water plus intraperitoneal injection of a vehicle, polyethylene glycol and glycerin (PEG); oral administration of water plus intraperitoneal injection of L-364,718 in PEG at a dose of 2 mg/kg; oral administration of camostat 400 mg/kg a day plus intraperitoneal injection of PEG, and oral administration of camostat plus intraperitoneal injection of L-364,718. Camostat or water alone was administered once a day beginning 1h after the last caerulein injection. L-364,718 or PEG alone was injected every 6h beginning 18h after the last caerulein injection. All observations were made on day 4. The rate of DNA synthesis was determined to evaluate pancreatic regeneration. L-364,718 inhibited regeneration whether or not camostat was given, while camostat given alone increased plasma CCK, promoted pancreatic DNA synthesis, and decreased the histologic severity of the pancreatitis. Histologic damage in the pancreas was milder in rats receiving both camostat and L-364,718 compared to those receiving only L-364,718. Therefore, we conclude that CCK participates in the regeneration of acinar cells after acute pancreatitis in rats, together with other factors.

Key words: L-364,718, camostat mesilate, caerulein-induced pancreatitis, regeneration, cholecystokinin

Introduction

Cholecystokinin (CCK) has been shown to stimulate pancreatic exocrine function and protein synthesis. Mainz et al reported that CCK shows a trophic effect on the normal pancreas, suggesting that CCK can stimulate pancreatic hyperplasia and hypertrophy. In addition, Hirata et al reported that CCK has hyperplastic effects not only under normal
circumstances but also following partial pancreatic resection. In our previous experiments, regeneration of acinar cells was enhanced by oral administration of camostat, a derivative of gabexate mesilate. Camostat has been shown to have therapeutic effects in patients with chronic pancreatitis and abdominal pain. In experimental animals, camostat increased CCK release from the proximal small intestinal mucosa, which increased the plasma CCK concentrations. Therefore CCK may participate in regeneration after acute pancreatitis. However, when a large dose of exogenous CCK is administered or the plasma CCK concentration is abnormally elevated, pancreatic exocrine secretion is suppressed and acute pancreatitis is induced.

Recently many new CCK receptor antagonists have been developed. According to several reports, these compounds have protective and therapeutic effects in various experimental models of acute pancreatitis. While CCK has been considered to exacerbate acute pancreatitis damage, a few reports have characterized regeneration after acute pancreatitis treated with CCK receptor antagonists. In the present study, L-364,718, a potent and specific CCK receptor antagonist, was used to elucidate the role of CCK during pancreatic regeneration in rats after caerulein-induced acute pancreatitis. L-364,718 competitively inhibits the binding of CCK with its receptors in rat pancreatic acinar cell membranes. In addition, this study used camostat which has been reported to convey a therapeutic benefit in rats with acute pancreatitis, an effect attributed to its action as a trypsin inhibitor. However, trypsin inhibitors can induce release of endogenous CCK in rats, and this CCK release in the early phase could aggravate acute pancreatitis. We therefore administered camostat in established caerulein-induced pancreatitis, either with or without L-364,718, to evaluate the effects on pancreatic regeneration.

Materials and Methods

**Experimental protocol (Fig. 1)**

Male Wistar rats weighing 190 to 210 g were maintained at 24°C in a room with a 12 h light-dark cycle. Rats had free access to water and a standard diet. To induce acute pancreatitis, caerulein (Kyowa Hakko Kogyo, Tokyo, Japan) dissolved in 2.5 ml of saline and was administered at a dose of 15 μg/kg by intraperitoneal (i.p.) injections hourly for 6 h. As treatment, either camostat mesilate (Ono Pharmaceutical, Osaka, Japan) dissolved in water or L-364,718 was injected intraperitoneally every 6 h beginning 18 h after the last caerulein injection. WP group: water plus PEG, WL group: water plus L-364,718, CP group: camostat plus PEG, CL group: camostat plus L-364,718. The same group designations apply in figures that follow, and in the Table.
in 1.0 ml of water at a dose of 400 mg/kg or water alone was administered via a gastric tube once a day at the same time for 4 days. The first dose was given 1 h after the last caerulein injection. L-364,718 (1.0 mg/ml) (Merck Sharp & Dome, West Point, PA, USA) was dissolved in a mixture of polyethylene glycol 400 and glycerin at a ratio of 11:0.6. Either L-364,718 at 2 mg/kg or PEG alone was administered via i.p. injection 4 times a day (at 6 hr intervals), beginning 18 h after the last caerulein injection. Following the induction of pancreatitis, rats (n=24) were randomly assigned to one of four groups: water plus PEG (WP group, n=6); water plus L-364,718 (WL group, n=6); camostat plus PEG (CP group, n=6); or camostat plus L-364,718 (CL group, n=6). On the 4th day after caerulein injection, when the rate of pancreatic DNA synthesis has been shown to peak in this model\(^2\), all rats were injected i.p with 9.25 MBq/kg of [methyl-\(^3\)H] thymidine (3.21 GBq/mmol; Amersham, Buckinghamshire, England) after overnight fasting. One hour after injection, rats were sacrificed and blood samples were collected. Plasma was separated and stored at \(-20\)\(^\circ\)C until measurement of CCK. The pancreas was rapidly removed and trimmed free of fat and lymph nodes on ice-cold plates.

**Biochemical determinations**

Protein content in pancreatic tissue was measured by the method of Lowry et al\(^{23}\). DNA was extracted from the pancreas by the Schmidt-Thannhauser-Schneider method\(^{24,25}\) and measured by a modification of the method of Burton\(^{26}\). \([\text{\(^3\)H}]\) thymidine incorporated into the DNA fraction was determined by scintillation counting. Specific radioactivity was expressed as incorporated radioactivity per microgram of DNA.

**Measurement of plasma CCK concentration**

Plasma CCK was extracted with Sep-Pak C\(_{18}\) cartridges as described by Liddle et al\(^{27,28}\), and measured by radioimmunoassay using a specific antibody against CCK (OAL-656) (Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan)\(^{29}\).

**Histologic examination**

A portion of the pancreas was fixed in 20\% neutral formalin buffered solution and

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Interstitial edema</th>
<th>Vacuolation</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP</td>
<td>2.33±0.78</td>
<td>1.17±0.39</td>
<td>0.92±0.67</td>
<td>0.92±0.67</td>
<td>5.33±2.02</td>
</tr>
<tr>
<td>WL</td>
<td>2.08±0.90</td>
<td>1.42±0.52</td>
<td>1.83±0.84</td>
<td>1.33±0.49</td>
<td>6.67±2.02</td>
</tr>
<tr>
<td>CP</td>
<td>1.17±0.72</td>
<td>0.75±0.45</td>
<td>0.50±0.52</td>
<td>0.17±0.39</td>
<td>2.58±1.56</td>
</tr>
<tr>
<td>CL</td>
<td>1.17±0.78</td>
<td>0.76±0.54</td>
<td>0.57±0.51</td>
<td>0.57±0.51</td>
<td>3.62±1.56</td>
</tr>
</tbody>
</table>

Group designations as for Fig. 1. The histologic grade for interstitial edema, vacuolation in acinar cells, inflammatory cell infiltration, and acinar cell necrosis was based on the percentage of affected acinar cells in one light microscopic field at X200; grade 0: 0\%; grade 1: < 5\%; grade 2: 5-25\%; grade 3: 25-50\%; grade 4: >50\%. Values are the mean±SD for six rats. **p<0.01.
embedded in paraffin. Sections were cut and stained with hematoxylin and eosin to be examined under a light microscope. The degree of interstitial edema, vacuolation in acinar cells, inflammatory cell infiltration, and acinar cell necrosis were graded in a blinded manner. The grades were defined as the percentage observed in one visual field at ×200 magnification as follows: grade 0: 0% ; grade 1: <5%; grade 2: 5-25%; grade 3: 25-50%; and grade 4: >50%. The scores were obtained by averaging five visual fields for each sample. Total scores were combined for the four variables as an index of severity of histologic changes. Additionally the mitotic index was determined as the percentage of acinar cells with mitotic nuclei among 2000 acinar cells observed.

**Autoradiography**

Tissue sections on slides were covered with an emulsion (Konica NR-M2; Konica, Tokyo, Japan) in darkness and kept at 4°C in a black box for 4 weeks. At the end of exposure, autoradiograms were developed and stained with hematoxylin. Using a light microscope, the labeling index of acinar cells was determined as a percentage of labeled cells among 2000 acinar cells.

**Statistical analysis**

All data are expressed as the mean ± SD. Significance was tested using one-way analysis of variance (ANOVA), with confirmation by the Scheffe's F-test when significant differences were found by ANOVA. Differences for which p value was less than 0.05 were defined as statistically significant.

**Results**

**Pancreatic protein level per DNA content**

Pancreatic protein level per DNA content, an indicator of cell size, was significantly higher in rats receiving camostat (CP, CL) than in the corresponding groups (WP and WL). In addition, pancreatic protein level per DNA content was significantly lower in rats receiving both L-364,718 and camostat (CL) than in rats receiving camostat alone (CP).

![Fig. 2. Pancreatic protein level per DNA content](image)

Protein content per microgram of DNA in groups administered camostat (CP, CL) was significantly higher than that in groups without camostat (WP, WL). Values are expressed as the mean ± SD for six rats. *p<0.05.
DNA synthesis in pancreatic tissue

Specific radioactivity in groups receiving L-364,718 (WL, CL) was significantly lower than that in the corresponding groups (WP, CP). Specific radioactivity in CP tended to be higher than that in WP, but no significant difference was seen between WL and CL or between WP and CP (Fig. 3).

Plasma CCK concentration

Plasma CCK concentration in groups receiving camostat (CP, CL) was significantly higher than that in the corresponding groups without camostat (WP, WL). No significant difference was

Pancreatic protein level per DNA content in WL was lower than that in WP, but this difference was not significant (Fig. 2).

DNA synthesis in pancreatic tissue

Specific radioactivity in groups receiving L-364,718 (WL, CL) was significantly lower than that in the corresponding groups (WP, CP). Specific radioactivity in CP tended to be higher than that in WP, but no significant difference was seen between WL and CL or between WP and CP (Fig. 3).

Plasma CCK concentration

Plasma CCK concentration in groups receiving camostat (CP, CL) was significantly higher than that in the corresponding groups (WP, WL). No significant difference was
Fig. 5. Histologic findings in the pancreas
A: Water plus PEG (WP group), B: Water plus L-364,718 (WL group),
C: Camostat plus PEG (CP group), D: Camostat plus L-364,718 (CL group)
Original magnification, ×200
In WP and WL, interstitial edema, vacuolation of acinar cells (arrows), and inflammatory cell infiltration (arrowheads) are observed. Inflammatory cell infiltration in WL is more severe than that in WP. In CP and CL, vacuolation of acinar cells (arrow) and inflammatory cell infiltration are slightly seen. The histologic severity of acute pancreatitis is alleviated in groups administered camostat, particularly in CP.

Light microscopic examination
Pancreatic tissue in WP showed interstitial edema, vacuolation of acinar cells, and inflammatory cell infiltration. Necrosis was present in some areas (Fig. 5A). In WL, acinar cell vacuolation and necrosis were observed and inflammatory cell infiltration was more severe than in WP (Fig. 5B). In CP, interstitial edema and inflammatory cell infiltration were not prominent, and little necrosis was present (Fig. 5C). In CL, acinar cell vacuolation and inflammatory cell infiltration were observed in some areas (Fig. 5D). The histologically defined total score was 5.33 ± 2.02 in WP, 6.67 ± 2.02 in WL, 2.58 ± 1.56 in CP, and 3.62 ± 1.56 in CL (Table 1). Histologic alterations in groups with L-364,718 (WL, CL) were more severe than those in the corresponding groups without L-364,718 (WP, CP), but no significant difference in total histologic score was seen between WP and
L-364,718 and Camostat in Acute Pancreatitis

The mitotic index in CP was significantly higher than that in CL. No significant difference was seen between WP and CP, or between WL and CL. Values are expressed as the mean ± SD for six rats. *p<0.05, **p<0.01.

Mitotic index

The mitotic index in CP (0.098±0.048%) was significantly higher than that in CL (0.029±0.032%; p<0.01). The mitotic index in CP was higher than that in WP (0.085±0.054%), but the difference was not significant. No significant difference was noted between WL (0.037±0.028%) and CL (Fig. 6).

Autoradiographic determination

The labeling index of acinar cells in WP (0.022±0.009%) or CP (0.027±0.005%) was...
significantly higher than that in the corresponding groups receiving L-364,718 (0.008 ± 0.003% in WL; 0.012 ± 0.005% in CL; p < 0.05). The labeling index in CP was higher than that in WP, but the difference was not significant. No significant difference was evident between WL and CL (Fig. 7).

Discussion

Administration of large doses of caerulein induces acute edematous pancreatitis in rats, representing a useful acute pancreatitis model because it is relatively easy to produce and the findings are consistent. In investigating the effects of a CCK receptor antagonist on pancreatic regeneration in this model, care is needed concerning timing of CCK receptor antagonist administration since caerulein is an analogue of CCK. Physiologic activity of caerulein could not be detected by bioassay in blood collected 30 min after administration to rats31). Histologically acute pancreatitis was evident at 12 h after the final caerulein injection, while acinar cell regeneration reached a peak on day 422, 32). Tanaka et al33) reported that the inhibitory effect of L-364,718 on pancreatic exocrine secretion persisted for at least 6 h at a dose of 2 mg/kg body weight, and that the effect of antagonist was similar with oral and intraperitoneal administration. Based on the results described above, the present study was designed to include L-364,718 injections every 6 h beginning 18 h after the last caerulein injection, while camostat administration began 1 h after the last caerulein injection. Subsequent evaluations were carried out when the rate of DNA synthesis was expected to peak (day 4). CCK has been shown to exacerbate acute experimental pancreatitis34-38), but later becomes an important factor in the regenerative phase. L-364,718 administered twice a day at 1.0 mg/kg for 13 days in rats with caerulein-induced pancreatitis was found to strongly inhibit spontaneous regeneration15). Pancreatic regeneration is also retarded in rats treated with CR-1505 after acute hemorrhagic pancreatitis16). The time point when the role of CCK changes from exacerbatory to pro-regenerative has not been strictly determined. In many reports where a CCK receptor antagonist proved effective in preventing or treating caerulein-induced pancreatitis5, 7-11, 13, 14), the CCK receptor antagonist was administered within 3 h of the last caerulein injection. In our present study with delayed administration, inflammatory changes of pancreatitis were histologically more severe in groups with L-364,718 treatment (WL, CL) than in groups without L-364,718 (WP, CP). No therapeutic effects from the CCK receptor antagonist were observed in our study. We used the rate of DNA synthesis, mitotic indices, and labeling indices of acinar cells as indicators of regeneration on day 4. In the group administered water and L-364,718 (WL), all indices were significantly lower, (i.e., regeneration was suppressed) compared with the group without L-364,718 (WP), presumably because of CCK-receptor blockade. An increased pancreatic histologic score also suggested suppression of regeneration. Rats receiving camostat orally and L-364,718 via intraperitoneal injection after caerulein-induced pancreatitis (CL) showed significantly less DNA synthesis (mitotic indices and labeling indices) in acinar cells than the group receiving camostat orally without L-364,718 (CP). Histologically, CL demonstrated a higher inflammatory score and fewer cells undergoing mitosis than CP, indicating suppression of regeneration. Pancreatic regeneration was strongly suppressed not only in WL but also in CL, suggesting that L-364,718 suppressed regeneration that otherwise would be enhanced by endogenous CCK release in response to camostat.
Camostat administration began 1 h after the last caerulein injection. Considering the negative feedback mechanism regulating pancreatic exocrine secretion, such early stimulation of CCK secretion might have been expected to worsen acute pancreatitis. However, the inflammatory score was lower in CP than in WP, while the total histologic score also showed a significant decrease in CP. Otsuki et al. reported that even a single administration of camostat, administered as early as 30 min after the final caerulein injection, reduced severity of pancreatitis. The group receiving both camostat and L-364,718 (CL) showed a somewhat milder inflammatory change than the group given L-364,718 alone (WL), suggesting that the effect of camostat upon caerulein-induced acute pancreatitis was mediated not only by regeneration of acinar cells through CCK, but also passively by other effects such as trypsin inhibition or enhanced release of endogenous secretin. Serine protease inhibitors can enter acinar cells, blocking activation of zymogens. Nakano et al. described pancreatic regeneration after caerulein-induced pancreatitis followed by treatment with CCK-8 and loxiglumide. They concluded that high doses of a CCK receptor antagonist inhibited pancreatic regeneration even when limited to the first 3 days following induction of pancreatitis. On the other hand, CCK-8 treatment after 3 days of loxiglumide administration greatly increased pancreatic protein, DNA, and enzyme content. CCK appears to play very different roles during the induction phase of acute pancreatitis and during the regeneration phase. How this shift occurs remains to be elucidated. Given in combination, a CCK receptor antagonist and protease inhibitor may promote pancreatic regeneration after acute pancreatitis. Our present observations demonstrate that there may be therapeutic benefit in administrating camostat for the pancreatic repair process. Further studies are needed to determine optimal timing in administering these agents.

Acknowledgements

We wish to thank Dr. Yoshio Hatta, former professor of the Second Department of Internal Medicine, Showa University School of Medicine, for his thoughtful advice with regard to the present study.

References

9) Murayama KM, Drew JB, Nahrwold DL and Joehl RJ: Cholecystokinin antagonist prevents hyperamylasemia


34) Evander A, Ihse I and Lundquist I: Influence of hormonal stimulation by caerulein on acute experimental

[Received December 27, 2002 : Accepted January 17, 2003]