Inhibition of nuclear factor- κ B activation improves the survival of rats with taurocholate pancreatitis

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Abstract

Background—Death in the early stages of severe acute pancreatitis is frequently the result of multiple organ dysfunction, but its mechanism is not clear.

Aims—To investigate the state of nuclear factor- κ B (NF- κ B) in macrophages of rats with lethal pancreatitis, and to assess the effectiveness of pyrrolidine dithiocarbamate, an inhibitor of NF- κ B, on the pathology and mortality.

Methods—Taurocholate pancreatitis was produced in rats, and the severity of the disease, the mortality, and activation of NF- κ B in peritoneal and alveolar macrophages were compared in rats receiving pyrrolidine dithiocarbamate (PDTC) treatment and those that were not.

Results—Taurocholate pancreatitis produced massive necrosis, haemorrhage, and severe leucocyte infiltration in the pancreas as well as alveolar septal thickening in the lung. NF- κ B was activated in peritoneal and alveolar macrophages six hours after pancreatitis induction. Pre-treatment with PDTC dose-dependently attenuated the NF- κ B activation and improved the survival of the rats, although it did not affect the early increase in serum amylase and histological findings.

Conclusions—Early blockage of NF- κ B activation may be effective in reducing fatal outcome in severe acute pancreatitis. (Gut 1999;44:253–258)

Keywords: pancreatitis; multiple organ dysfunction; nuclear factor- κ B; pyrrolidine dithiocarbamate; macrophages; rat

product.^{11 12} It is present in the cytoplasm in an inactive homo- or hetero-dimeric form coupled to an inhibitor I κ B, but a number of stimuli degrade I κ B and activate NF- κ B. The unbound NF- κ B translocates to the nucleus and binds to specific *cis* elements located in the promoters of various genes. There is good evidence that NF- κ B plays a key role in the control of cytokine induced expression of many immune and inflammatory genes.¹³ In septic patients, it has been shown that NF- κ B is activated in peripheral blood mononuclear cells.¹⁴ In rat endotoxaemia models, the therapeutic effectiveness of *N*-acetylcysteine and pyrrolidine dithiocarbamate (PDTC), inhibitors of NF- κ B activation, has been reported.^{15 16} Because NF- κ B regulates the expression of a variety of inflammatory genes such as tumour necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, and inducible nitric oxide synthase (iNOS) and because these inflammatory genes are activated at the transcriptional level during severe acute pancreatitis, NF- κ B may also play a role in the development of systemic complications during this disease. The objectives of this study were to evaluate the state of NF- κ B activation in a lethal pancreatitis model and to assess the effect of PDTC on the pathology and mortality.

Materials and methods

PRODUCTION AND EVALUATION OF TAUROCHOLATE (TCA) PANCREATITIS

Male Wistar rats weighing 200–250 g were maintained at 23°C on a 12 hour light/dark cycle and allowed free access to water and standard laboratory chow. From 12 hours before the start of the experiments, the animals were deprived of food but were allowed access to water. This study was conducted with the consent of the ethics committee for the use of experimental animals of the Tohoku University School of Medicine.

TCA pancreatitis was produced by the method of Aho *et al.*¹⁷ Under pentobarbital anaesthesia (50 mg/kg body weight), a laparotomy was performed and 5% sodium taurocholate (1 ml/kg body weight) was injected into the biliopancreatic duct at a rate

The most serious complication during severe acute pancreatitis is the occurrence of multisystem organ failure (MOF) during the early stages. Mortality from acute pancreatitis is closely related to the development of early systemic complications, and the death rate of patients with such pathological conditions has been reported to reach 20–50%.¹ Several mediators such as activated pancreatic enzymes,^{2 3} cytokines,^{4 5} endotoxins,⁶ superoxides,⁷ and arachidonate metabolites^{8–10} have been suggested to play important roles in the pathogenesis, but as the mechanism of MOF in severe acute pancreatitis is still unclear, it remains difficult to develop an effective therapy.

Nuclear factor- κ B (NF- κ B) is a member of the Rel family of transcriptional regulatory proteins including p50, p52, p65, c-Rel, Rel B, and the *Drosophila* morphogen dorsal gene

Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; IL, interleukin; iNOS, inducible nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; MOF, multisystem organ failure; NF- κ B, nuclear factor- κ B; PBS, phosphate buffered saline; PDTC, pyrrolidine dithiocarbamate; TCA, taurocholate; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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Figure 1 Histological findings for the pancreas (A), lung (B), and liver (C) 24 hours after the induction of taurocholate (TCA) pancreatitis. Original magnification $\times 40$.

of 0.2 ml/min by a microinfusion pump (Minipuls 3; Gilson Medical Electronics, Villiers le Bel, France). Controls received an intraductal infusion of saline (0.2 ml/min). The rats recovered from the anaesthesia and were allowed access to water. Then 12 or 24 hours after the operation, the rats were killed by an overdose of pentobarbital sodium and blood was collected by cardiac puncture. In some rats, the lung, liver, and a segment of the pancreas close to the spleen were removed 24 hours after the induction of TCA pancreatitis. The tissues were fixed by immersion in 4% paraformaldehyde and embedded in paraffin wax. Sections (3 μ m thick) were cut, deparaffinised, and stained with haematoxylin and eosin for histological examination. Serum amylase activity was measured by the blue-starch method using a Phadebas amylase test.¹⁸ The packed cell volume was determined by centrifuging the blood-aspirated glass capillaries at 2700 rpm.

EFFECTS OF PDTC ON MACROPHAGE NF- κ B ACTIVATION AND SURVIVAL OF THE RATS

Peritoneal and alveolar macrophages were obtained from the control rats and from those

with TCA pancreatitis 12 hours after the induction of pancreatitis, and NF- κ B activation was studied by electrophoretic mobility shift assay (EMSA). PDTC is a potent inhibitor of NF- κ B.^{19,20} To evaluate the effect of PDTC, various doses (1, 10, or 100 mg/kg) were injected intraperitoneally one hour before the induction of TCA pancreatitis, and NF- κ B activation in the macrophages was studied. Serum amylase activity and packed cell volume were examined as described above.

The effects of PDTC on the mortality of the rats with TCA pancreatitis were assessed for seven days. Firstly, the dose-dependency of the effects on mortality was examined by injecting various doses (1, 10, or 100 mg/kg) of PDTC intraperitoneally one hour before the induction of pancreatitis. To evaluate the effects of the timing of PDTC administration on the mortality, the dose of PDTC was fixed at 10 mg/kg and it was injected intraperitoneally one hour or just before, or two or six hours after the induction of TCA pancreatitis.

PREPARATION OF PERITONEAL AND ALVEOLAR MACROPHAGES

In control rats, 10 ml of cooled phosphate buffered saline (PBS; 0.02 M, pH 7.2) was injected into the abdominal cavity and recovered. In the rats with TCA pancreatitis, the ascites was obtained through a puncture. The abdominal fluids were centrifuged at 800 rpm for 15 minutes, and the sedimented cells were designated peritoneal inflammatory cells. These cells were then seeded in a polymethylpentene dish (100 $\times$ 15 mm; Nalge Company, New York, New York, USA) and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, streptomycin (100 μ g/ml), and penicillin (100 units/ml) at 37°C for two hours. Non-adherent cells were removed by several washes with PBS, and adherent cells were harvested using a rubber "policeman". Over 95% of these cells were judged to be macrophages by immunohistochemical staining using monoclonal anti-rat monocyte/macrophage IgG (Serotec, Oxford, UK).

Alveolar macrophages were harvested by bronchoalveolar lavage. Using a plastic syringe inserted into the trachea, 5 ml pyrogen-free PBS was injected into the lung and was gently aspirated. The bronchoalveolar lavage fluid was centrifuged at 800 rpm for 15 minutes to sediment the cell components. About 95% of the sedimented cells were judged to be macrophages by the method mentioned above.

EMSA OF NF- κ B

Activation of NF- κ B was examined by EMSA.^{12,13} The oligonucleotide 5'-CCAACTGGGGACTCTCCCTTTGGGA-3' (κ B), which corresponds to the κ B binding site (-85 to -76) of macrophage iNOS promoter^{21,22}, was synthesised and end-labelled with [γ -³²P]ATP (Amersham, Tokyo, Japan) using T₄ polynucleotide kinase (Takara Biomedicals, Otsu, Japan). After the oligonucleotide and the complementary oligonucleotide had been annealed, the ³²P labelled double stranded oligonucleotide obtained was used as a specific

Figure 2 Electrophoretic mobility shift assay of NF- κ B using the 32 P labelled probe. Peritoneal macrophages (A) and alveolar macrophages (B) were collected from rats with taurocholate pancreatitis at the indicated time after the induction of pancreatitis.

Figure 3 Competition assays of the activated NF- κ B. Peritoneal macrophages were collected 12 hours after the induction of taurocholate pancreatitis. Nuclear extracts were preincubated with no reagents (lane 1), a 10-fold (lane 2) or a 100-fold (lane 3) molar excess of unlabelled κ B oligonucleotide, or a 100-fold molar excess of mutant κ B oligonucleotide (lane 4). In the competition assay, an excess of the oligonucleotide competitor was preincubated with the nuclear extract for 60 minutes at room temperature.

probe. For the competition assay, the unrelated oligonucleotide 5'-CCAACTGCTCACTTC TCCCTTTGGGA-3' (κ B-mu), which lacked the κ B binding site, and its complementary oligonucleotide were annealed, and used as a non-specific probe. Nuclear proteins were extracted from macrophages as described by Schreiber *et al.*²³ and the protein content was determined using an assay kit (BCA protein assay reagent; Pierce, Rockford, Illinois, USA). The nuclear extract equivalent to 5 μ g protein was incubated with 2×10^5 cpm radiolabelled probe in reaction buffer (10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 3 μ g poly(dI-dC), 75 mM KCl, 1 mM dithiothreitol). The mixture was then subjected to electrophoresis on 5% polyacrylamide gel at 150 V in 0.25 $\times$ Tris/borate buffer. After being dried, the gel was exposed to x ray film overnight. The specificity of the EMSA was confirmed by adding the following to the nuclear extract: a 10-fold or 100-fold molar excess of unlabelled oligonucleotide (competi-

tion assay), a 100-fold molar excess of mutant oligonucleotide, and 2 μ g/ml each of the rabbit polyclonal antibody against p50, p65, c-Rel, and p52 subunit of NF- κ B (supershift assay). The radioactive bands were quantified using a BAS 2000 image analyser (Fuji Film Co., Tokyo, Japan).

CHEMICALS

TCA and PDTC were purchased from Sigma Chemical Co, St Louis, Missouri, USA. The Phadebas amylase test was from Pharmacia Diagnostic, Uppsala, Sweden. The purified polyclonal rabbit antibodies against p50, p65, c-Rel, and p52 were purchased from Santa Cruz Biotechnology Inc, Santa Cruz, California, USA. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

DATA ANALYSIS

Every experiment was carried out in at least six rats, and representative results are presented for the EMSA studies. Serum amylase activity and packed cell volume for control rats, TCA pancreatitis rats, and PDTC-pretreated TCA pancreatitis rats are expressed as mean (SEM) for 16 rats, and the data were assessed by unpaired Student's *t* test. Mortality was evaluated by the log rank method. Statements referring to a significant difference indicate a *p* value of 0.05 or less.

Results

Intraductal administration of TCA produced haemorrhagic necrotising pancreatitis with massive haemorrhage, severe infiltration of inflammatory cells, and large areas of necrosis in the pancreas (fig 1A). In the lung tissue of these rats, the alveolar interstitium was considerably thickened by the aggregation of leucocytes (fig 1B). However, no particular changes were observed in the histology of the liver (fig 1C).

Figure 4 Supershift assays of activated NF- κ B. Peritoneal macrophages (lanes 1–5) and alveolar macrophages (lanes 6–10) were collected 12 hours after the induction of taurocholate pancreatitis. Nuclear extracts were preincubated with no reagents (lanes 1 and 6), or with 2 μ g each of the rabbit polyclonal antibody against p50 (lanes 2 and 7), p65 (lanes 3 and 8), c-Rel (lanes 4 and 9), or p52 (lanes 5 and 10). In the supershift assays, incubation with the respective antibody was carried out at 4°C for 60 minutes.

In the peritoneal macrophages, translocation of NF- κ B to the nuclei, which indicated activation of NF- κ B, had increased six hours after the induction of TCA pancreatitis and continued to increase thereafter (fig 2A). Activation of NF- κ B was also observed in the alveolar macrophages with a similar time course, although it was slightly weaker than in the peritoneal macrophages (fig 2B). The densities of the bands at six and 12 hours after the induction of pancreatitis relative to that at 0 hours were 3.3 and 6.7 in the peritoneal macrophages and 1.4 and 2.8 in the alveolar macrophages respectively. The specific activation of NF- κ B was confirmed by both the competition assay (fig 3) and the supershift assay (fig 4). Because the bands corresponding to NF- κ B were supershifted by adding either the antibody against p50 (fig 4, lanes 2 and 7) or that against p65 (fig 4, lanes 3 and 8), and since the gel shift retardation did not occur after the addition of the antibody against c-Rel (fig 4, lanes 4 and 9) or that against p52 (fig 4, lanes 5

and 10)), the major component of NF- κ B in the nuclei was assumed to be a heterodimer of p50 and p65.

Rats were treated with different doses (1, 10, or 100 mg/kg) of PDTC one hour before the intraductal injection of TCA, and NF- κ B activation, serum amylase activity, and packed cell volume were examined 12 hours after the induction of pancreatitis. NF- κ B activation in the peritoneal as well as alveolar macrophages was dose-dependently inhibited by PDTC (fig 5). The increase in packed cell volume was inhibited by PDTC, but neither the gross pathological appearance of the pancreas (fig 6) nor the increase in serum amylase (table 1) was affected at all.

About 75% of the rats with TCA pancreatitis had died within three days of the induction of pancreatitis, and 88% had died within seven days. Administration of PDTC one hour before the induction of TCA pancreatitis improved the mortality in a dose-dependent manner. When PDTC at 10 mg/kg was given, 56% of

Figure 5 (A) Effects of pyrrolidine dithiocarbamate (PDTC) on NF- κ B activation. Peritoneal macrophages (lanes 1–4) and alveolar macrophages (lanes 5–8) were obtained 12 hours after the induction of taurocholate pancreatitis. Lanes 2–4 and 6–8 show NF- κ B activation in the respective type of macrophages obtained from rats pretreated with the indicated doses of PDTC (1, 10, and 100 mg/kg) one hour before the induction of pancreatitis, whereas lanes 1 and 5 show NF- κ B activation in the respective type of macrophages obtained from rats not pretreated with PDTC. (B) Specificity studies of activated NF- κ B in the peritoneal macrophages of pancreatitis rats pretreated with 1 mg/kg PDTC. Nuclear extracts from macrophages were preincubated with no reagents (lane 1), with a 100-fold molar excess of unlabeled κ B oligonucleotide (lane 2), with 2 μ g/ml of the rabbit polyclonal antibody against either p50 (lane 3) or p65 (lane 4). Then the mixtures were subjected to electrophoretic mobility shift assay.

Figure 6 Effects of pyrrolidine dithiocarbamate (PDTC) on the histological findings in the pancreas. PDTC at a dose of 10 mg/kg was given intraperitoneally one hour before the induction of pancreatitis and the tissues were obtained 24 hours after the induction of pancreatitis. Original magnification $\times 40$.

Figure 7 Effects of pyrrolidine dithiocarbamate (PDTC) on the mortality of the rats with taurocholate pancreatitis. PDTC was given intraperitoneally one hour before the induction of pancreatitis, and mortality was examined for seven days after the induction of pancreatitis. * $p < 0.05$ compared with the rats not given treatment.

the rats were still alive seven days after the induction of pancreatitis (fig 7). To evaluate the therapeutic effect of PDTC, the dose of PDTC was fixed at 10 mg/kg and given before or after the induction of pancreatitis. When PDTC was given intraperitoneally just before the induction of pancreatitis, the survival was improved to a degree similar to that when given one hour before the induction of pancreatitis. However, PDTC was less effective when it was given two hours after the induction of pancreatitis, and no favourable effect was observed when it was given six hours after the induction of pancreatitis (fig 8).

Table 1 The effects of pyrrolidine dithiocarbamate (PDTC) on serum amylase and packed cell volume in rats with taurocholate (TCA) pancreatitis

	Serum amylase after 12 hours (IU/l)	Packed cell volume (%)	
		12 h	24 h
Control	10 228.0 (723.1)	43.5 (2.1)	44.5 (2.5)
TCA pancreatitis			
Without PDTC	19 434.2 (2117.7)	56.5 (3.2)	59.6 (3.3)
With PDTC	18 476.4 (1906.7)	49.5 (2.3)*	46.5 (4.8)*

Results are expressed as mean (SEM).

Rats were treated with or without 10 mg/kg PDTC one hour before the induction of TCA pancreatitis. Control rats received intraductal infusion of saline as described in experimental design. The number of rats in each group was 16.

* $p < 0.05$ compared with no PDTC (unpaired Student's t test).

Figure 8 Effect of timing of pyrrolidine dithiocarbamate (PDTC) (10 mg/kg) administration on mortality. * $p < 0.05$ compared with the rats not given treatment.

Discussion

In the present study, we have shown that NF- κ B in the peritoneal and alveolar macrophages are activated as early as six hours after the induction of TCA pancreatitis, and that in vivo administration of PDTC before the onset of pancreatitis inhibits dose-dependently the activation of NF- κ B in these cells. Inhibition of NF- κ B by PDTC was accompanied by significant improvements in mortality. Dunn *et al*²⁴ have recently reported that the NF- κ B DNA binding activity in the extracts of the pancreas increased after the onset of experimental acute pancreatitis, and that inhibition of NF- κ B activation produced a decrease in the pancreatitis induced increase in serum amylase. In this study, no significant differences were found in the increase in serum amylase or the histology of the pancreas between the PDTC treated and untreated rats. The important inference from our results is that the beneficial effects of PDTC on mortality in the normally lethal experimental pancreatitis are not the result of improvement in the inflammatory processes of the pancreas, but may be caused by suppression of the processes related to induction of systemic organ dysfunction. These results strongly indicate that certain mechanisms activate the generalised monocyte/macrophage systems during the early phase of severe acute pancreatitis. Although no significant improvement in mortality was obtained when PDTC was given after the induction of pancreatitis, the inhibition of NF- κ B activation very early in the pathological course may suggest new therapeutic approaches for reducing death from systemic complications in severe acute pancreatitis.

The binding sites for NF- κ B are found in the promoters of a variety of genes of proinflammatory molecules, such as IL-6, IL-8, TNF- α , and iNOS, cell surface adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), as well as cell surface mediators, such as major histocompatibility complexes class I and II.^{11,12} In a previous report, we showed that iNOS was expressed in the peritoneal macrophages of TCA pancreatitis rats, but not in those of caerulein pancreatitis rats.²⁵ We have also recently found that the ascitic fluid of TCA pancreatitis rats could

selectively upregulate the expression of ICAM-1 and VCAM-1 but not E-selectin in cultured human umbilical vein endothelial cells, and that ICAM-1 was indeed expressed in vivo in the pulmonary vascular endothelial cells during TCA pancreatitis (unpublished results). These results strongly suggest that certain soluble mediators that can activate macrophages and vascular endothelial cells are released into the ascites during inflammation of the pancreas, and that these mediators may circulate to further activate macrophages and vascular endothelial cells in distant organs. This idea may be supported by the present finding that NF- κ B activation in alveolar macrophages showed a time course similar to that of peritoneal macrophages during TCA pancreatitis.

It has recently been shown that knockout mice deficient in IL-1 type 1 receptors, TNF type 1 receptors, or both IL-1 and TNF type 1 receptors showed greatly improved survival after the induction of lethal pancreatitis.²⁶ It has also been shown that inactivation of IL-1 converting enzyme by an inactivator or by knockout improved the survival of animals with lethal necrotizing pancreatitis.²⁷ Therefore TNF- α and IL-1 β may play key roles in the development of systemic complications during severe acute pancreatitis.^{28,29} In addition to cytokines, it has recently been shown that lysophosphatidylcholine, a product of phospholipase A₂, can induce ICAM-1 expression in vascular endothelial cells.³⁰ Circulating endotoxins, which may be produced through bacterial translocation during pancreatitis, may be another candidate for mediation of MOF.^{31,32} As all these soluble mediators are activators of NF- κ B in macrophages and vascular endothelial cells, NF- κ B may be the central point at which multiple signals from various different mediators converge.^{11-13,19} Our results indicate that blocking NF- κ B activation in the very early stages of the processes may be an effective approach to preventing death from MOF during severe acute pancreatitis.

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