ROLE OF CYTOKINES IN EXPERIMENTAL ACUTE PANCREATITIS

Ph.D. Thesis

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Contents

List of publications

I.
Farkas, Gy., Marton, J., Mandi, Y., and Szederkenyi E.

Surgical strategy and management of infected pancreatic necrosis

British Journal of Surgery, 1996; 83: 930-933.

II.
Marton , J., Farkas, Gy., Takacs, T., Nagy, Z., Szasz, Z., Varga, J., Jarmay, K.,.Balogh, A., Lonovics, J.

Beneficial effects of pentoxifylline treatment of experimental acute pancreatitis in rats

Research in Experimental Medicine (accepted for publication)

III.
Farkas, Gy., Marton, J., Nagy, Z., Mandi, Y., Takacs, T., Deli, M. A., Abraham, C. S.

Experimental acute pancreatitis results in increased blood-brain barrier permeability in the rat: a potential role for tumor necrosis factor and interleukin 6

Neuroscience Letters, 1998; 242: 3, 147-150.

IV.
Farkas, Gy., Mandi, Y., and Marton J.

Modification of cytokine production in septic condition following necrotizing pancreatitis

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V.
Marton, J., Farkas, Gy., Nagy, Z.,. Takacs, T., Varga, J., Szasz, Z., Balogh, A., and J. Lonovics

Plasma levels of TNF and IL-6 following induction of acute pancreatitis and pentoxifylline treatment in rats

Acta Chirurgica Hungarica, 1997; 36 (1-4): 223-225

VI.
Farkas, Gy., Nagy, Z., Marton, J., Mandi, Y.

Relevance of cytokine production to infected pancreatic necrosis

Acta Chirurgica Hungarica, 1997; 36 (1-4): 86-88

VII.
Márton, J., Farkas, Gy., Szederkényi, E. és Avramov, K.

Az akut nekrotizáló pancreatitis szeptikus szövődményének kezeléséről

Orvosi Hetilap, 1995; 136 (17): 893-895.

VIII.
Márton, J., Farkas, Gy., Nagy, Zs., Takács, T., Jármay, K., Varga, J. és Balogh, Á.

Cytokinek kisérletes akut pancreatitisben

Orvosi Hetilap, 1997; 138 (12): 739-742.

1. INTRODUCTION

Acute pancreatitis remains a great challenge for both the medical team and the health service, in spite of the application of novel surgical and intensive therapeutic methods. Its symptoms range from a mild, self-limiting disease to multiple-organ failure and sepsis. The mortality rate for oedematous pancreatitis is very low, but for necrotizing pancreatitis it has been reported to reach from 30% to 80%. In the early stages the outcome is often unpredictable (Allerdyce 1987; Exley *et al.* 1992; Imrie 1997). Despite numerous clinical and experimental investigations the pathogenesis and the factors contributing to the progression of the disease are poorly understood. There is little correlation between the predisposing factors and the outcome of the underlying disease. It is accepted worldwide that different pathogenetic factors produce similar inflammatory events and these events lead to the activation of a final common pathway of acute pancreatitis, but the explanation of different outcomes remains obscure (Gross *et al.* 1993; McKay *et al.* 1994). The overwhelming majority of the patients present with acute pancreatitis due to alcohol abuse or gallstone disease.

Claude Bernhard pioneered research into experimental pancreatitis, producing acute pancreatitis by injecting bile and olive oil into the canine pancreatic duct in 1856. Much research has been performed to create a simple, cheap and reproducible experimental model which is relevant to human conditions (Lerch 1994). A wide range of experimental models of acute pancreatitis have been proposed (secretagogue hyperstimulation, thediet-induced, the closed duodenal loop technique, the pancreatic ductal perfusion/infusion model, the duct ligation-induced, vascular occlusion model and *ex vivo* models). These different models represent various degrees of severity of acute pancreatitis, due to the different stimuli and the destruction of the pancreas. It is extremely important to bear in mind that experimental findings can not be extrapolated *in toto* to human pancreatitis. (Thanks to the clinical efforts, mild pancreatitis can be treated sufficiently in a conservative way: intravenous fluid replacement, nasogastric suction, analgetic drugs, *etc.*) Acute necrotizing pancreatitis and infected pancreatic necrosis followed by sepsis and multiple-organ failure are

currently the leading causes of death in acute pancreatitis (Farkas et al. 1996). We have selected the taurocholic acid-induced model for our investigations, this model having a degree of severity similar to that of human acute necrotizing pancreatitis (Aho *et al.* 1980).

Numerous authors have pointed out that various drugs proposed for the treatment of acute pancreatitis produced excellent results under experimental conditions, but failed to furnish a marked beneficial effect in a clinical setting. This fact could be explained by the difference between the human disease and the experimental model or delayed treatment after the onset of acute pancreatitis in humans. There is growing evidence that major surgical intervention, trauma, burns and sepsis cause excessive leukocyte activation and the overproduction of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-6 (IL-6) (Grewal *et al* 1994; Farkas 1995). This cascade of events can lead to the development of a systemic inflammatory response and multiple-organ failure, independently of the origin of the process. The common features of the systemic inflammatory response and severe acute necrotizing pancreatitis reasonably suggest that cytokines, and especially TNF, may play a pivotal role in the pathogenesis of the above-mentioned processes.

Cytokines are low molecular weight soluble protein molecules which play a crucial role in the communication between cells during the activation and amplification of inflammatory responses (Lowry 1993; Galley and Webster 1996). Cytokines are extremely active at very low concentrations, combining with small numbers of high-affinity cell surface receptors. The number of known cytokines is still growing.. TNF exerts a wide variety of effects, due to its ability to mediate the expression of genes. It plays an important role in host resistance to infection, as an immunostimulant and mediator of the inflammatory response, and it promotes haematopoesis. IL-6 induces hepatic acute phase protein synthesis, effects on B-cells (differentiation and antibody production) and T-cells (activation, proliferation, differentiation and IL-2 production induction), the formation of adhesion molecules and haematopoiesis (Molloy *et al.* 1993; Blackwell and Christman 1996).

Patients with acute pancreatitis may develop changes in consciousness and pancreatic encephalopathy (Boon *et al.* 1991; Estrada *et al.* 1979; Ott *et al.* 1994; Wang *et al.* 1995; Winsler *et al.* 1990). Increased serum concentrations of pro-inflammatory cytokines have been reported to be associated with central nervous system (CNS)

damage in some diseases, including acquired immunodeficiency syndrome (AIDS) (Mintz *et al.* 1989), malaria (Beutler and Grau 1993) and multiple sclerosis (Sharief and Thomson 1992). Elevated serum and cerebrospinal fluid levels of IL-6 were found in patients with AIDS-related encephalopathy or systemic lupus erythematosus with CNS involvement. In contrast, severe head trauma induces significant increases in the serum TNF and IL-6 concentrations (Ott *et al.* 1994). *In vivo* (Abraham *et al.* 1996; Beutler and Grau 1993; Brett *et al.* 1996, Megyeri *et al.* 1992; Salja *et al.* 1995) and *in vitro* experimental data (Deli *et al.* 1995; de Vries *et al.* 1996), and the results of clinical studies (Beutler and Grau 1993; Feuerstein *et al.* 1994; Sharief and Thomson 1992) indicated that both TNF and IL-6 can open the blood-brain barrier (BBB) .It is presumed that pathological conditions inducing the production of these cytokines (e.g. acute pancreatitis) may result in vasogenic brain oedema formation.

Because of the central role of TNF in the systemic inflammatory response and acute necrotizing pancreatitis, it seems reasonable that those drugs which can inhibit pro-inflammatory cytokine production should influence the severity and outcome of acute necrotizing pancreatitis. Two thoroughly investigated drugs, that are used worldwide, pentoxifylline and octreotide, were selected for our investigations.

Pentoxifylline, 1-(5-Hydroxycyclohexyl)-3,7-dimethylxanthine has been used extensively for the treatment of patients with intermittent claudication by virtue of its rheologic action. It has marked effects not only on blood viscosity and flow, but also on some other properties. Pentoxifylline decreases platelet aggregation and adhesions, decreases fibrinogen, and stimulates the release of prostacyclin I_2, tissue plasminogen activator and antithrombin III. It influences wound healing by increasing the production of fibroblast collagenases and decreasing the production of collagen, fibronectin and glycosaminoglycan (Samlaska and Winfield1994). Pentoxifylline increased the rate of neutrophil migration and attenuated meningitis, peritonitis and Gram-negative sepsis in animal models. Besides these beneficial properties, pentoxifylline can inhibit the TNF production of monocytes and suppresses leukocyte stimulation by TNF. Other investigations have revealed that pentoxifylline inhibits the transcription of messenger RNA of TNF (Doherty *et al.* 1991) and can prevent the toxic effect of TNF on endothelial cells and suppress the natural killer cell activity.

Due to this complex pattern of immunologic properties, pentoxifylline has strong immunomodulatory effects and is a promising agent in TNF-related diseases.

Octreotide is a synthetic octapeptide analogue of somatostatin, with similar pharmacological effects, but with a considerably enhanced duration of action and pharmacological potency. It is a neurotransmitter in the central nervous system. It is a hormone which regulates insulin, glucagon, vasoactive intestinal peptide, secretin, *etc.* It is widely distributed in the gastrointestinal tract, and has a marked inhibitory/regulatory effect on gastrointestinal and pancreatic endocrine and exocrine secretion. It also reduces gastrointestinal motility, prolongs the transit time and increases water and electrolyte absorption. Because of their inhibitory action on pancreatic secretion, somatostatin and octreotide have been investigated worldwide in acute pancreatitis (Grosman and Simon 1990). The experimental studies have revealed a beneficial effect on the serum amylase level, but the improvements in pancreatic histology and survival rates are controversial (Schwedes *et al.* 1979; Baxter *et al.* 1985; Zhu *et al.* 1991). Most clinical studies have demonstrated a trend towards an improved survival and a lower complication rate, but the numbers of patients were generally too small for the findings to be conclusive, or the classification and degree of severity of the pancreatitis were different or not comprehensive. Reviews of clinical studies have emphasized the uncertainty of the classification of pancreatitis in different patient populations (Buchler *et al.* 1994; Luengo *et al.* 1994; McKay *et al.* 1997). Some studies have stressed the importance of reducing the complication rate following pancreatic surgery (Farkas *et al.* 1993). One of the most promising areas of application of somatostatin and octreotide is for the prevention of post-ERCP pancreatitis. Some investigations have revealed a significant improvement in the severity of post-ERCP pancreatitis (Bordas *et al.* 1988). The theoretical basis of application of octreotide in acute pancreatitis is its significant inhibitory effect on basal and stimulated pancreatic enzyme secretion, but the importance of enzyme secretion in severe acute necrotizing pancreatitis remains questionable. Other studies have emphasized the influence of somatostatin on eicosanoid synthesis (van Ooijen *et al.* 1992) and on the function of the reticulo-endothelial system (Baxter *et al.* 1985), but little attention has been paid to the effect of octreotide on cytokine production in acute necrotizing pancreatitis.

2. AIMS OF THE STUDY:

There is growing evidence that activation of the cytokine cascade is the key event of systemic inflammatory processes, including trauma, burns, sepsis. Severe acute necrotizing pancreatitis can produce clinical symptoms which are highly similar to the above-mentioned pathologic conditions. Therefore, the aims of the study reported here:

To select a suitable experimental model which is comparable to human necrotizing pancreatitis. (comparable severity of histologic changes, systemic complications, mortality rate, *etc.*).

To determine the TNF and IL-6 levels after the induction of acute necrotizing pancreatitis and t investigate the rates of change of these cytokines.
To investigate the correlation between the severity of pancreatitis and the changes in the TNF and IL-6 levels.

To measure the effects of cytokines (especially TNF and IL-6) on the blood brain barrier.
To investigate the roles of these cytokines in the development of vasogenic brain oedema.

To investigate the effects of pentoxifylline on cytokine production and the severity of pancreatitis.

To determine the effects of octreotide on cytokine production and the severity of pancreatitis.

3. MATERIALS AND METHODS

3.1. Animals

Male Wistar rats weighing 200 to 260 g were housed in wire-bottom cages and fed standard rat chow and water *ad libitum* (n = 297). The experimental procedures followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the local ethical committee.

3.2. Induction of acute pancreatitis

Acute necrotizing pancreatitis was induced in accordance with Aho *et al.* (1980). Aether anaesthesia was induced, the abdomen was shaved, prepped and draped in a sterile fashion and a midline incision was made. The pancreatic duct was cannulated transduodenally and the common bile duct was temporarily closed with a metal clamp. A knot was tightened around the pancreatic duct and the cannula. Then, 150 µl or 200 µl of 4% or 6% taurocholic acid (REANAL, Hungary) was injected via the cannula during 1 minute. After infusion, the clamp, the knot and the cannula were removed and the duodenal wound was closed with a single figure-of-eight 6-0 prolene suture. Sham-operated animals underwent laparotomy and exploration of the duodenum and pancreas. Another group of animals received 200 µl of 6% taurocholic acid subcutaneously. Animals were killed after 6, 24, 48 or 72 hours.

3.3. Laboratory tests

Analysis of plasma samples: All blood samples were centrifuged at 2000 rpm for 30 minutes immediately after collection. Serum amylase levels were determined by means of the Phadebas test and are reported in standard units (Ceska *et al.* 1969).

Wet pancreatic weight to body weight ratios (pw/bw) were calculated and are reported in mg/g. TNF was titrated in a bioassay on cell line WEHI-164 (Espevik and Niessen-Meyer 1986).

IL-6 was measured via its proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (Arden *et al.* 1987). The activities were calibrated against rm TNF (GENZYME, Cambridge, England) and rm IL-6 (Sigma-Aldrich, Munich), respectively.

3.4. Histology

The pancreas fragments were fixed in 10% neutral formaldehyde solution, embedded in paraffin and stained with haematoxylin and eosin, and with Crossmon's trichrome for light microscopy. The different histologic lesions were scored in accordance with Hughes *et al.*(1996) on 3 rats at each time point.

3.5. Blood-brain barrier permeability

Four experimental groups were formed: untreated control animals 0 hours (n = 6) and 3 groups of rats with acute pancreatitis 6 hours (n = 8), 24 hours (n = 6) or 48 hours (n = 9) after the induction.

The development of vasogenic brain oedema as the extravasation of two BBB permeability tracers (both from Sigma): sodium fluorescein (SF, mw: 376) and Evans blue-labelled albumin (EBA, mw: 67 000), was measured in all 4 groups of rats, as was previously described (Ábrahám et al. 1996). A solution of both dyes in isotonic saline (2%, 5ml/kg) was given in an intravenous injection 30 minutes before the end of the experiments. Intravascular dyes were removed by perfusion with 200 ml/kg isotonic saline. Sera and tissue samples from the parietal cortex, hippocampus, striatum cerebellum and medulla of rats were homogenized in 3.0 ml of cold 7.5% trichloroacetic acid and centrifuged at 10 000 g for 10 minutes. The EBA (absorbance: 620 nm) and SF (excitation 440 nm, emission: 525 nm) concentrations of supernatants were analysed with a Hitachi F2000 fluorimeter as described by Ábrahám *et al.* (1996). Extravasation was expressed as brain tissue concentration divided by serum concentration for both dyes.

3.6. Effect of pentoxifylline treatment.

The pentoxifylline-treated group received 7 mg/kg of pentoxifylline intraperitoneally at the time of operation and 6, 12, or 24 hours later. Treated and untreated animals and sham-operated animals were killed 6, 24, 48 or 72 hours after the operation.

3.7. Effect of octreotide treatment

The octreotide-treated group received 1 μg (4 μg/kg) of octreotide (Novartis) subcutaneously immediately after the induction of pancreatitis and 24 or 48 hours later. The control group (200 μl of taurocholic acid intraductally) and the sham-operated group received isotonic saline subcutaneously. Treated, control and sham-operated animals were killed 6, 24, 48 or 72 hours after the operation.

Analysis of peritoneal fluid samples: Before blood sampling, during the induction of anaesthesia, 5 ml of isotonic saline was injected into the abdominal cavity. After the abdominal incision, peritoneal fluid was aspirated and tested for TNF and IL-6.

3.8. Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed with the Statgraphics program (STSC INC. Statistical Graphics Corporation, two-sample analysis), with the Student *t* test. *P* values less than 0.05 were accepted as significant. The values were compared between groups by using either one-way analysis of variance (ANOVA) or the Kruskal-Wallis ANOVA on ranks. Multiple comparisons were performed with either the Student-Newman-Keuls test or Dunn's method. The differences were considered significant at $P < 0.05$ (blood-brain permeability).

4. RESULTS

4.1. Induction of pancreatitis

The serum amylase levels were significantly elevated in every group receiving taurocholic acid intraductally (23 200±2 754, 22 157±3 492, 15 226±2 247, 11 266±622 U/ml) as compared to the sham-operated group (393±402 and 283±116 U/ml). Taurocholic acid given subcutaneously did not cause raised amylase levels. The wet pancreatic weight to body weight ratios were significantly elevated after the intraductal administration of 6% taurocholic acid. There was no mortality in the sham-operated group or in the group which received taurocholic acid subcutaneously. There was no mortality in the group receiving 200 µl of 4% taurocholic acid or 150 µl of 6% taurocholic acid, but the mortality in the group receiving 200 µl of 6% taurocholic acid was 43% after 48 hours.. These facts could be explained in terms of the severity of the acute necrotizing pancreatitis. We did not observe any morphologic or laboratory sign of acute pancreatitis after the subcutaneous administration of taurocholic acid.

The histologic analysis of the pancreata revealed interstitial oedema and necrosis of the pancreatic acinar cells in the early phase (at 6 hours), and invasion of neutrophil leukocytes and microabscess formation in the late phase (at 48 hours). The lung histology showed congestion and perivascular oedema in the early phase, and neutrophil leukocyte infiltration and hyaline membrane formation in the late phase. Hyaline membrane formation can be considered an early sign of respiratory distress syndrome.

TNF was detected in the early phase (at 6 hours) after the injection of 150 µl of 6% taurocholic acid (35.7±9.9 U/ml), but not at 24 or 48 hours. The elevated TNF levels 24 and 48 hours after the injection of 200 µl of 6% taurocholic acid (35.0±5.0 and 36.6±6.0 U/ml) can be regarded as a consequence of severe acute necrotizing pancreatitis.

The IL-6 levels remained elevated at 48 hours after the administration of 200 µl of 6% taurocholic acid (6 728±3 442 pg/ml). In the event of the lower concentration or volume of taurocholic acid, the IL-6 levels decreased rapidly after 48 hours

(612.5±477 and 535.5±227.5 pg/ml, respectively). To summarize these initial results, we concluded that this model is suitable for the characterization of acute necrotizing pancreatitis. The mortality attained 43% at 48 hours, and histologic analysis demonstrated severe pathologic changes. The TNF level remained elevated, while the IL-6 level decreased slowly, in accordance with the severity of the acute necrotizing pancreatitis.

4.2. Blood-brain barrier permeability

Taurocholic acid-induced acute necrotizing pancreatitis resulted in typical symptoms, and some of the animals died within a few days. In the preliminary experiments, the mortality rate was found to be 11% at 6 hours, 32% at 24 hours, and 43% at 48 hours. In the present study, the pancreatic weight/body weight ratio was significantly elevated ($P < 0.05$) in taurocholic acid-treated treated rats throughout the experimental period. The serum amylase levels were also increased in the treated animals, but a tendency to decrease was suggested at 48 hours. The serum TNF concentration was significantly elevated at 6 and 24 hours, as were the IL-6 levels at 24 and 48 hours as compared with the values measured in the control animals. We found a time-dependent BBB opening for both tracers in the brain regions observed during acute pancreatitis. A significantly ($P < 0.05$) increased SF extravasation was seen at 6 h in hippocampus, cerebellum and medulla, but this phenomenon disappeared later. An elevated BBB transport for albumin was seen in each brain region after 6 and 24 hours of acute pancreatitis, except for the parietal cortex at 6 hours.

4.3. Effect of pentoxifylline treatment

Pentoxifylline treatment did not change pw/bw significantly either. The increase in the serum amylase activity was significant in the pentoxifylline-treated group ($19.2\pm2.9\times10^3$ and $16.9\pm3.9\times10^3$ U/l) and in the control group ($12.5\pm4.6\times10^3$ and $22.5\pm5.1\times10^3$ U/l) as compared with the sham-operated group at 6 and 24 hours, but somewhat lower at 48 and 72 hours. There was no major difference in amylase level between the control group and the pentoxifylline group. The histological scores were determined for the control group and the pentoxifylline-treated group at various time-points. The scoring results indicated that at 48 hours after the induction of pancreatitis the severity of the acinar necrosis was more expressed in the control group (score: 1.5

= lobular necrosis over more than 30% of the surface area, with microabscesses), while in the pentoxifylline-treated group at the same time point, focal acinar cell necrosis was detected with mild pancreatic inflammation (score: 0.66). TNF was not detected at any time in the sham-operated group. The TNF level was elevated in the pentoxifylline-treated group (54.10±20 and 10.9±4.2 U/ml) at 6 and 24 hours, but TNF was not detectable at 48 hours. The TNF levels was elevated in the control group at 6, 24 and 48 hours (30.2±5.4, 35.0±5.0 and 36.6±6.0 U/ml, respectively).There was no detectable amount of TNF in any group at 72 hours. The IL-6 levelwas elevated in the pentoxifylline-treated group at 6 hours and 24 hours (6 463±1 307 pg/ml, 10 329±5 571 pg/ml) but significantly decreased at 48 and 72 hours (137.5±85.5 and 71.4±188 pg/ml) as compared with the control group (8 014±2 793 and 7 083±2 844 pg/ml, respectively). The mortality at 48 hours was 43% in the control group and 11% in the pentoxifylline-treated group.

4.4. Effect of octreotide treatment

Octreotide administration significantly decreased the pancreatic weight to body weight ratio at 24 and 48 hours as compared with the control group (5.17±0.49 and 4.97±0.59 mg/g *versus* 7.94±1.05 and 7.86±0.48 mg/g, respectively). The serum amylase levels were significantly lower in the octreotide-treated group than in the control group at 24, 48 and 72 hours (5 512±794, 1 045±210 and 971±198 U/l *versus* 22 592±5 172, 5 007±1 199 and 7 383±1 307 U/l, respectively). The histologic lesions were scored in accordance with Hughes *et al.* (1996) at each time-point. Oedema, vascular changes, inflammation, acinar necrosis, fat necrosis, calcification and fibrosis were scored. Surprisingly, none of these parameters improved significantly in the octreotide-treated group as compared with the control group. The serum TNF levels were elevated in the control group at 6, 24 and 48 hours, while that at 72 hours was zero. The peritoneal fluid TNF level increased slowly, but remained high at 48 and 72 hours (40.55±49.65 and 27.97±48.93 U/ml). The serum TNF levels were decreased significantly in the octreotide-treated group as compared with the control group at 6, 24 and 48 hours (0.57±1.51, 2.0±3.34 and 0 U/ml *versus* 50±15.49,. 37.5±18.37 and 13.13±12.51 U/ml, respectively). The IL-6 levels in the serum in the octreotide-treated group and in the control group were elevated at 6 hours and were enormously high in the peritoneal

fluid at 6 hours (80 000±43 817 *versus* 58 500±33 335 pg/ml). These elevated IL-6 levels had decreased rapidly by 24, 48 and 72 hours. There was no significant difference in IL-6 production between the octreotide-treated group and the control group. The mortality rate at 48 hours was 43% in the control group and 5% in the octreotide-treated group.

5. DISCUSSION

Taurocholic acid given intraductally can elevate the amylase levels and increase the pancreatic weight to body weight ratio.
Histologic analysis of the pancreas proved severe acute necrotizing pancreatitis with microabscess formation, and beginning respiratory distress syndrome was observed in the lung.
The TNF and IL-6 levels increased significantly after administration of 200 µl of 6% taurocholic acid. The mortality was high (43% at 48 hours).
To summarize our initial results, we concluded that the acute necrotizing pancreatitis induced by 200 µl of 6% taurocholic acid is similar to the human one in severity and in the pathologic changes.

The severity of acute pancreatitis may be characterized by the extrapancreatic complications (renal, cerebral, hepatic and pulmonary dysfunction). The survival of the patients depends not only on the damage of the pancreas, but also on the amplitude and duration of the immuno-inflammatory response. Cytokines form a network of proteins and lipids and interact with each other via either inhibition or stimulation, and result in a very differentiated and redundant regulation of inflammatory and immune reactions. Leukocyte immigration into the pancreas, and the induction of TNF and IL-6 synthesis are the early events of acute necrotizing pancreatitis. The pancreatic concentrations of TNF and IL-6 are about two or three times higher than the serum concentrations (Schölmerich 1996). Elevated serum cytokine concentrations reflect the overproduction of these mediators in the pancreas, and later in other affected organs. Attenuation or inhibition of the activated cytokine cascade is a very promising concept. Due to the redundant regulation of the inflammatory response it is not likely that any single drug will be 100% effective. Better results can be achieved with combinations of different agents.

In the present study, a time-dependent increase in BBB permeability was seen during taurocholic acid-induced acute pancreatitis in rats. Wang *et al.* (1995) recently found an increased brain tissue water content, but no leakage of serum albumin in acute

pancreatitis 12 hours after the intraductal infusion of bile. In our study, the development of vasogenic brain oedema correlated with increases in serum cytokine levels. However, TNF seems to be a more important mediator in this respect, because the induction of TNF preceded that of IL-6, and the peak of serum TNF level was seen in the first 24 hours, during which the increased BBB permeability was detected.

Pancreatic encephalopathy is a severe complication of acute pancreatitis, the incidence of which, according to clinical data, is 11-35% of all cases (Boon *et al.* 1991, Estrada *et al.* 1979, Saez and Royula-Gonzalo 1989). Its aetiology is still unknown. The pathogenetic role of alcohol withdrawal and hyperamylasaemia has been suggested, but it has not been confirmed (Boon *et al.* 1991; Estrada 1979). Vasogenic brain oedema, *i.e.* the extravasation of serum components to the brain interstitial fluid, is presumed to contribute to the development of brain injury in acute pancreatitis. Previous studies indicated that cytokines may be responsible for the multiple-organ failure in acute pancreatitis (Gross *et al.* 1993; Mándi *et al.* 1995; Norman *et al.* 1995). We assume that the corresponding serum cytokine concentrations may also result in cerebral oedema formation in patients with acute pancreatitis.

The significant increases in serum amylase level demonstrated that the pancreas was affected in both the control group and the pentoxifylline-treated group. Pentoxifylline treatment did not decrease the serum amylase level relative to that in the control group. These facts suggest that pentoxifylline does not influence the early events of taurocholic acid-induced acute pancreatitis and the autodigestion of the pancreas. The TNF level decreased rapidly after 6 hours in the pentoxifylline-treated group. The lack of a detectable TNF level in the sham-operated group indicates that laparotomy itself is not as serious an injury as the taurocholic acid-induced acute pancreatitis. The rapid decrease in IL-6 level in the pentoxifylline-treated group as compared with the control group is very similar to the change in TNF.

Pentoxifylline treatment is not able to protect the pancreas from acute damage (elevated serum amylase level, and elevated TNF and IL-6 in the early phase). The histologic results also indicate that pentoxifylline administration does not prevent the acute damage to the pancreas, but it can modify the severity of the inflammatory processes of the pancreas in this experimental model. The lower mortality rate (11%

in the pentoxifylline-treated group *versus* 43% in the control group) lends support to this concept. Via its blocking effect on TNF production and attenuation of the activation of the cytokine cascade, pentoxifylline may be beneficial in the complex treatment of acute necrotizing pancreatitis.

In accordance with the experimental data, we routinely use pentoxifylline in the treatment of patients presenting with acute necrotizing pancreatitis and a septic condition. We observed impressive clinical results after pentoxifylline administration (Farkas *et al.* 1995, Mándi *et al.* 1995)

The principle of octreotide treatment in acute pancreatitis is based on its inhibitory effect on pancreatic secretion. However, the importance of the inhibition of stimulated or basal pancreatic secretion in severe acute necrotizing pancreatitis remains questionable. When the pancreatic acinar cells are destroyed and digested, and the pancreatic blood flow is decreased, there is little chance for normal or stimulated pancreatic secretion (Kusterer *et al.* 1992). On the other hand, different clinical and experimental studies have demonstrated quite clearly that, in mild or moderate form of acute pancreatitis, the inhibition of pancreatic secretion has a beneficial effect (Steinberg and Schlesselman. 1987; De Rai *et al.* 1988; Hoffmann *et al.* 1996, Kaplan *et al.* 1996, Paran et al. 1996,Tulassay *et al.* 1995). An inhibitory effect on pancreatic secretion was also observed in our study (the serum amylase level decreased significantly after octreotide treatment). Surprisingly, we did not detect a significant improvement in the histologic score in the octreotide-treated group. This could be explained by the severity of the physical and mechanical destruction of the pancreatic gland during the induction of pancreatitis. However, the improved survival at 48 hours (5% in the octreotide-treated group *versus* 43% in the control group) suggests the efficacy of octreotide treatment. In recent years, numerous data have accumulated which stress the crucial role of cytokines (especially TNF) in the pathogenesis of acute necrotizing pancreatitis and the development of extrapancreatic complications. In most of the studies based on the serum cytokine levels, however, little attention has been paid to the cytokine production of the peritoneal macrophages. We concluded that continuously elevated TNF levels in the peritoneal fluid reflect the stimulation of the peritoneal macrophages. An elevated IL-6 level in the peritoneal fluid (especially at 6 hours) may be considered a good marker of the stimulation of the peritoneal macrophages, but we could not demonstrate a significant change in the peritoneal IL-6

level after octreotide treatment. The overproduction of TNF can provoke a systemic inflammatory response and the systemic (extrapancreatic) manifestation of pancreatitis. The decreased amylase and TNF levels and the improved survival rate observed in our study support the concept of the value of octreotide treatment in acute pancreatitis. It is noteworthy that octreotide treatment inhibited not only pancreatic secretion, but also TNF production in acute necrotizing pancreatitis. This anti-inflammatory effect may contribute to its complex beneficial effect in the treatment of acute pancreatitis.

In accordance with the experimental data, we gularly use octreotide in the clinical practice after complicated pancreatic operations, and observe improvement in the postoperative conditions of the patient (Farkas *et al.* 1993).

6. CONCLUSIONS

Taurocholic acid-induced acute pancreatitis is a simple, cheap and reproducible experimental model.

The mortality rate in this experimental model is comparable with that in the human disease.

The production and serum levels of TNF and IL-6 correlate with the severity of necrosis and the systemic inflammatory process.

TNF and IL-6 may contribute to vasogenic brain oedema formation during acute pancreatitis.

Pentxifylline treatment may be beneficial in the complex treatment of acute necrotizing pancreatitis (decreased mortality rate, decreased cytokine production and improvement in the histology).

Due to its complex effect, octreotide can partially ameliorate the deleterious consequences of acute necrotizing pancreatitis (decreased mortality rate and decreased cytokine production).

Abstracts and other publications related to the Ph.D. thesis

Rosner, E., Farkas, Gy., Márton, J.:
A thermolabilis amylase jelentősége az akut pancreatitis kórlefolyásában (Abstract)
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BENEFICIAL EFFECT OF OCTREOTIDE TREATMENT IN ACUTE PANCREATITIS IN RATS

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SUMMARY

Conclusions: Octreotide treatment contributes to the regulation of TNF production in sodium taurocholate-induced acute necrotizing pancreatitis in rats. Due to its complex effect, octreotide can partially ameliorate the deleterious consequences of acute necrotizing pancreatitis. Elevated TNF and IL-6 levels in the peritoneal fluid may be considered a consequence of the activation of peritoneal macrophages.
Background: The effects of octreotide on the exocrine pancreatic function have been investigated in numerous studies, but a little attention has been paid to its influence on the cytokine production in acute pancreatitis.
Methods: Acute pancreatitis was induced by the retrograde injection of taurocholic acid into the pancreatic duct in male Wistar rats. The serum amylase activity, the wet pancreatic weight/body weight ratio, and the TNF and IL-6 levels were measured. Four μg/kg octreotide was administered subcutaneously at the time of induction of pancreatitis and 24, 48 hours later. Rats were sacrificed 6, 24, 48 or 72 hours after the operation.
Results: The serum amylase level and pancreatic weight to body weight ratio were decreased significantly in the octreotide-treated group. The serum TNF level was decreased significantly in the octreotide-treated group as compared to the control group at 6, 24 and 48 hours (0.57±1.51, 2.0±3,34, and ∅ versus 50±15.49, 37.5±18.37 and 13.125±12.51 U/ml, respectively). The ascites TNF level was decreased to zero in the octreotide-treated group and was elevated in the control group at 72 hours (27.97±48.93 U/ml). Il-6 production in ascites was extremely high in both groups at 6 hours (80 000±43 817 pg/ml and 58 500±33 335 pg/ml) but the difference was not significant.

Key words: octreotide, acute pancreatitis, tumor necrosis factor-α, interleukin-6,

INTRODUCTION

Somatostatin and octreotide exert marked inhibitory and regulatory effects on gastrointestinal and pancreatic endocrine and exocrine secretion. Because of their inhibitory action on pancreatic secretion, somatostatin and octreotide have been investigated worldwide in acute pancreatitis (1). The experimental studies have revealed a beneficial effect on the serum amylase level, but the improvements in pancreatic histology and survival rates are controversial (2,3,4). Most clinical studies have demonstrated a trend toward an improved survival and a lower complication rate, but the numbers of patients were generally too small for the findings to be conclusive, or the classification and degree of severity of pancreatitis were different or not comprehensive. Reviews of clinical studies have emphasized the uncertainty of classification of pancreatitis in different patient populations (5,6,7,8). Some studies have stressed the importance of reducing the complication rate following pancreatic surgery (9). One of the most promising areas of application of somatostatin and octreotide is for the prevention of post-ERCP pancreatitis. Some studies have revealed a significant improvement in the severity of post-ERCP pancreatitis (10). The theoretical basis of application of octreotide in acute pancreatitis is its significant inhibitory effect on basal and stimulated pancreatic enzyme secretion, but the importance of enzyme secretion in severe acute necrotizing pancreatitis remains questionable. Another study has emphasized the influence of somatostatin on eicosanoid synthesis (11) and on the function of the reticulo-endothelial system(12). The roles of endothelin and endothelin antagonists have also been investigated in the pathogenesis of acute necrotizing pancreatitis (13). There is growing evidence that octreotide can modify the production of different cytokines especially tumor necrosis

factor-α (TNF) and interleukin-6 (IL-6) (14, 15, 16). The systemic cytokine response has been investigated thoroughly, but little attention has been paid to the cytokine production of peritoneal macrophages (17). The present study was designed to investigate the effect of octreotide on cytokine production in acute necrotizing pancreatitis, to measure the cytokine levels in the intraperitoneal fluid, and to compare the changes in the ascites and serum cytokine levels with time.

MATERIALS AND METHODS

Male Wistar rats weighing 200-260 g were used in all experiments. The animals were kept at a constant room temperature of 27C, with free access to water and standard laboratory chow (LATI, Gödöllő, Hungary). The experiment followed the Principles of laboratory animal care of NIH. Acute necrotizing pancreatitis was induced in accordance with Aho et al. (18). Ether anesthesia was induced, the abdomen was shaved, prepped and draped in a sterile fashion, and a midline incision was made. The pancreatic duct was cannulated transduodenally and the common bile duct was temporarily closed with a metal clamp. A knot was tightened around the pancreatic duct and the cannula. Next, 200 µl 6% taurcholic acid (Reanal, Hungary) was injected via the cannula during 1 minute. After infusion, the clamp, knot and cannula were removed and the duodenal wound was closed with a single figure-of-eight 6-0 prolene suture. Sham-operated animals underwent laparotomy and exploration of the duodenum and pancreas. The octreotide-treated group received 1 µg (4 µg/kg) Sandostatin (Novartis) subcutaneously immediately after the induction of pancreatitis and 24 or 48 hours later. The control group (200 µl tarocholic acid intraductally) and

the sham-operated group received isotonic saline subcutaneously. Treated, control and sham-operated animals were killed 6, 24, 48 or 72 hours after the operation. Wet pancreatic weight to body weight ratios (pw/bw) were calculated and are reported in mg/g.

Analysis of plasma samples: All blood samples were centrifuged at 2 000 rpm for 30 minutes immediately after collection. Serum amylase levels were determined by means of the Phedebas test (19) and are reported in standard units. TNF was titrated in a bioassay on cell line WEHI-164 (20). IL-6 was measured via its proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (21). The activities were calibrated against rm TNF (Genzyme, Cambridge, England) and rm IL-6 (Sigma-Aldrich, Munich, Germany).

Analysis of peritoneal fluid samples: Before blood sampling, during the induction of anesthesia, 5 ml isotonic saline was injected into the abdominal cavity. After the abdominal incision, peritoneal fluid was aspirated and tested for TNF and IL-6.

Histological examinations: The pancreas fragments were fixed in 10% neutral formaldehyde solution, embedded in paraffin and stained with hematoxylin and eosin, and with Crossmon's trichrome for light microscopy. The different histologic lesions were scored in accordance with Hughes (22) on 3 rats at each time point.

Statistical analysis: Results are expressed as means±SEM. Statistical analysis was performed with the Student *t* test. P values less than 0.05 were accepted as significant.

RESULTS

Octreotide administration significantly decreased the pancreatic weight to body weight ratio at 24 and 48 hours as compared to the control group (5.17±0.49 and 4.97±0.59 mg/g versus 7.94±1.05 and 7.86±0.48 mg/g respectively). The serum amylase levels were significantly lower in the octreotide-treated group than in the control group at 24, 48 and 72 hours (5512±794, 1045±210, 971±198 U/l versus 22592±5172, 5007±1199 7383±1307 U/l, respectively). The histologic lesions were scored in accordance with Hughes at each time point. Edema, vascular changes, inflammation, acinar necroris, fat necrosis, calcification and fibrosis were scored. Surprisingly, none of these parameters improved significantly in the octreotide-treated group as compared to the control group. The serum TNF levels were elevated in the control group at 6, 24 and 48 hours, while that at 72 hours was zero. The peritoneal fluid TNF level increased slowly, but remained high at 48 and 72 hours (40.55±49.65 and 27.97±48.93 U/ml). The serum TNF levels were decreased significantly in the octreotide-treated group as compared to the control group at 6, 24 and 48 hours (0.57±1.51; 2.0±3.34 and ∅ U/ml versus 50±15.49;. 37.5±18.37 and 13.13±12.51 U/ml, respectively). The IL-6 levels in the serum in the octreotide-treated group and in the control group were elevated at 6 hours and were enormously high in the peritoneal fluid at 6 hours (80000±43817 versus 58500±33335 pg/ml). These elevated IL-6 levels decreased rapidly by 24, 48 and 72 hours. There was no significant difference in IL-6 production between the octreotide-treated and the control group. The mortality rate at 48 hours was 43% in the control group and 5% in the octreotide-treated group.

DISCUSSION

The principle of octreotide treatment in acute pancreatitis is based on its inhibitory effect on pancreatic secretion. However, the importance of the inhibition of stimulated or basal pancreatic secretion remains questionable in severe acute necrotizing pancreatitis. When the pancreatic acinar cells are destroyed and digested and the pancreatic blood flow is decreased, there is little chance for normal or stimulated pancreatic secretion (23). In the other hand, the different clinical and experimental studies demonstrate quite clearly that in mild or moderate form of acute pancreatitis the inhibition of pancreatic secretion has a beneficial effect (24,25,26,27,28,29). An inhibitory effect on pancreatic secretion was also observed in our study (the serum amylase level decreased significantly after octreotide treatment). Surprisingly, we did not detect a significant improvement in the histologic score in the octreotide-treated group. This could be explained by the severity of the physical and mechanical destruction of the pancreatic gland during the induction of pancreatitis. However, the improved survival at 48 hours (5% in the octreotide-treated group versus 43% in the control group) suggests the efficacy of octreotide treatment. In recent years, numerous data have accumulated which stress the crucial role of cytokines (especially TNF) in the pathogenesis of acute necrotizing pancreatitis and the development of extrapancreatic complications. In most of the studies based on the serum cytokine levels, however, little attention has been paid to the cytokine production of the peritoneal macrophages. We concluded that continuously elevated TNF levels in the peritoneal fluid reflect the stimulation of the peritoneal macrophages. An elevated IL-6 level in the peritoneal fluid (especially at 6 hours) may be considered a good marker of the stimulation of the peritoneal macrophages, but we could not demonstrate a significant change in the peritoneal IL-6 level after octreotide treatment. The

overproduction of TNF can provoke a systemic inflammatory response and the systemic (extrapancreatic) manifestation of pancreatitis. The decreased amylase and TNF levels and the improved survival rate observed in our study support the concept of the value of octreotide treatment in acute pancreatitis. It is noteworthy that octreotide treatment inhibited not only pancreatic secretion but also TNF production in acute necrotizing pancreatitis. This antiinflammatory effect may contribute to its complex beneficial effect in the treatment of acute pancreatitis.

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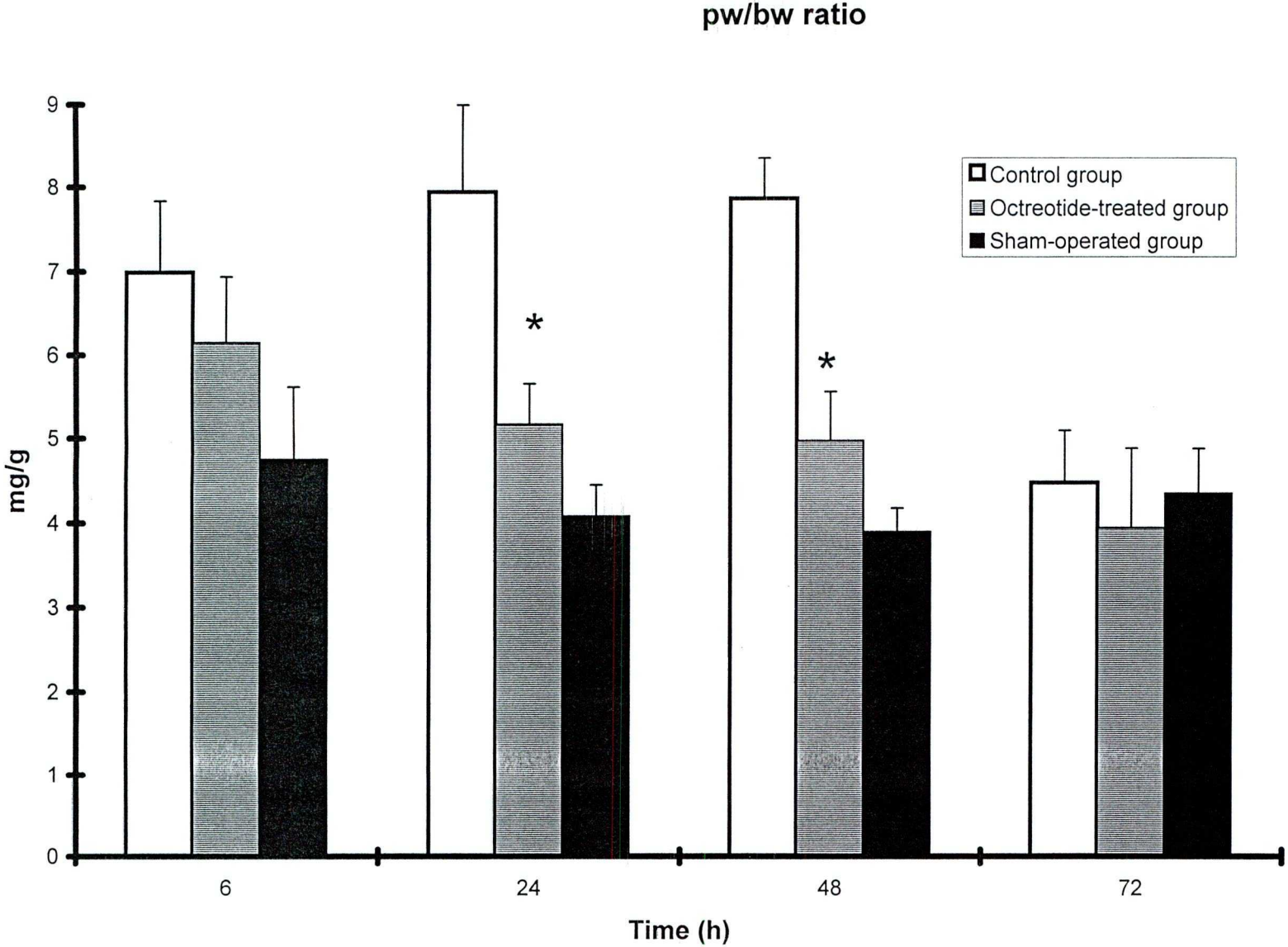
Legends to Figures

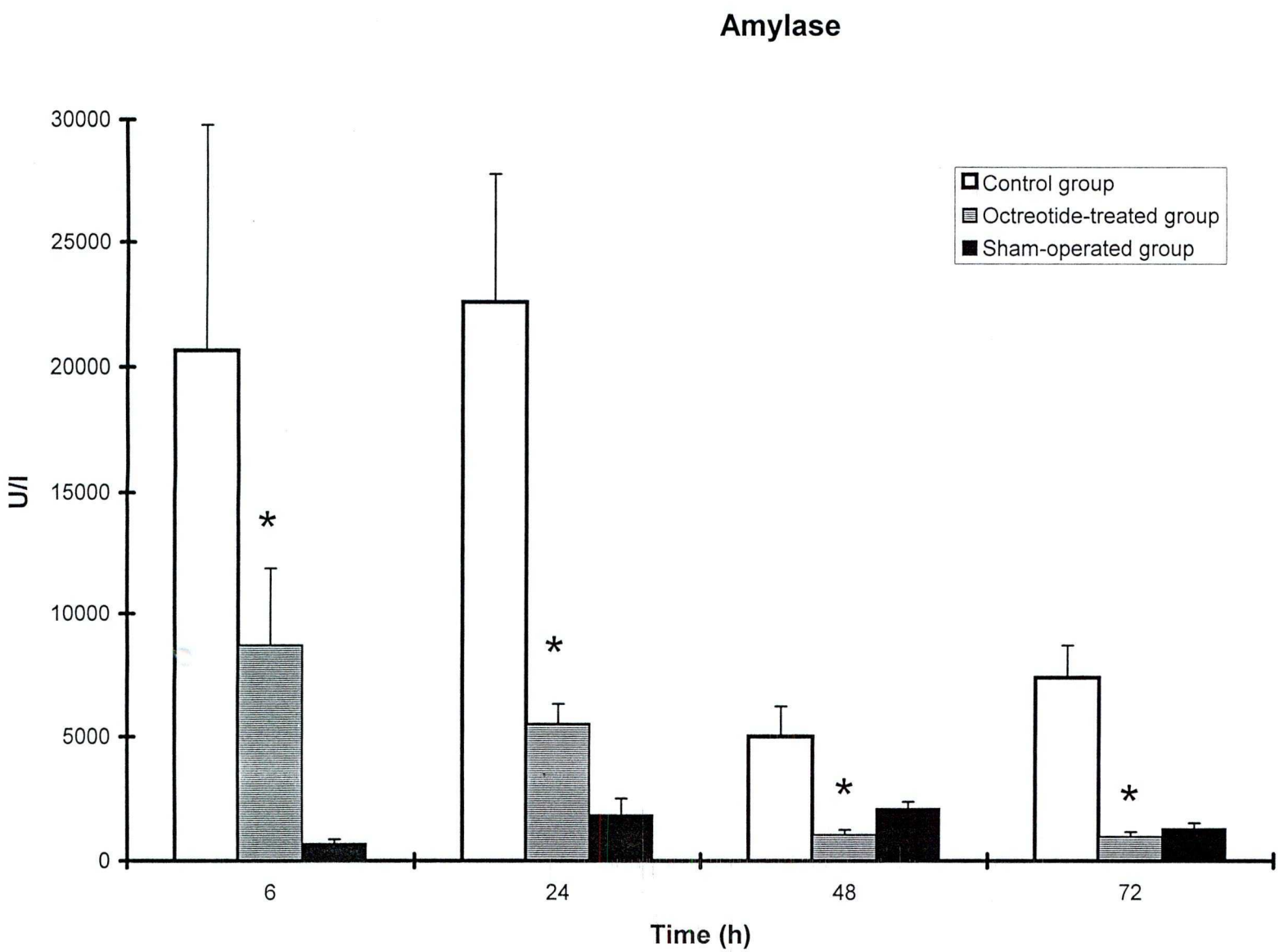
Fig. 1. Ratio pw/bw in taurocholic acid (200 µl 6%)-induced acute pancreatitis in rats. The octreotide-treated group (taurocholic acid intraductally + octreotide subcutaneously), the control group (taurocholic acid intraductally and isotonic saline subcutaneously) and the sham-operated group of animals were surgically prepared and killed as described in the Materials and Methods section. The pancreatic weight and body weight were measured and the ratio pw/bw was calculated. Values are means ±SEM for groups of 6 rats.

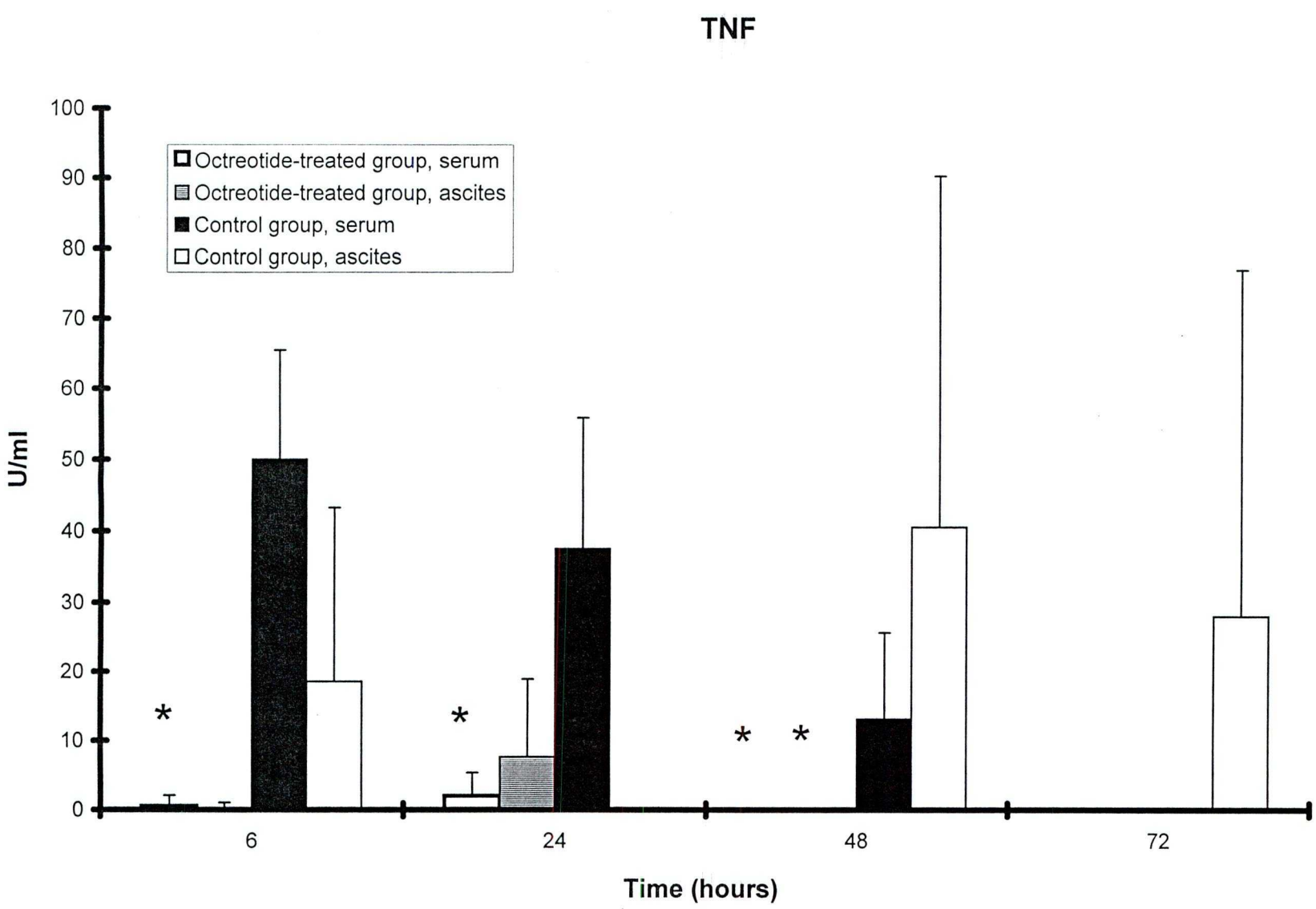
Fig. 2. Serum amylase activity in taurocholic acid (200 μl 6%)-induced acute pancreatitis in rats. The octreotide-treated group (taurocholic acid intraductally + octreotide subcutaneously), the control group (taurocholic acid intraductally and isotonic saline subcutaneously) and the sham-operated group of animals were surgically prepared and killed as described in the Materials and Methods section. Values are means ±SEM for groups of 6 rats.

Fig. 3. Changes in serum and peritoneal fluid TNF levels with time in taurocholic acid (200 μl 6%)-induced acute pancreatitis in rats. The octreotide-treated group (taurocholic acid intraductally + octreotide subcutaneously), the control group (taurocholic acid intraductally and isotonic saline subcutaneously) and the sham-operated group of animals were surgically prepared and killed as described in the Materials and Methods section. TNF was titrated in a bioassay on cell line WEHI-164. The activites were calibrated against rm TNF (GENZYME, Cambridge, England). Values are means ±SEM for groups of 6 rats.

Fig. 4. Changes in serum and peritoneal fluid IL-6 levels with time in taurocholic acid (200 μl 6%)-induced acute pancreatitis in rats. The octreotide-treated group (taurocholic acid intraductally + octreotide subcutaneously), the control group (taurocholic acid intraductally and isotonic saline subcutaneously) and the sham-operated group of animals were surgically prepared and killed as described in the Materials and Methods section. IL-6 was measured via its proliferative action on the IL-6-dependent mouse hybridoma cell line B-9. The activites were calibrated against rm IL-6 (Sigma-Aldrich, Munich, Germany). Values are means ±SEM for groups of 6 rats.


pw/bw ratio
Control group
Octreotide-treated group
Sham-operated group
mg/g
0
1
2
3
4
5
6
7
8
9
*
*
6
24
48
72
Time (h)


Amylase
30000
25000
20000
15000
10000
5000
0
U/l
Control group
Octreotide-treated group
Sham-operated group
*
*
*
*
6
24
48
72
Time (h)


TNF
Octreotide-treated group, serum
Octreotide-treated group, ascites
Control group, serum
Control group, ascites
U/ml
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
6
24
48
72
Time (hours)

IL-6