The role of glucocorticoid-dependent mechanism in the pathogenesis of experimental acute pancreatitis

Ph.D.Thesis

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LIST OF ARTICLES AND ABSTRACTS RELATED TO THE SUBJECT OF THE DISSERTATION

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Paszt Attila, Takács Tamás, Rakonczay Zoltán, Kaszaki József, Wolfard Antal, Tiszlavicz László, Lázár György, Duda Ernő, Szentpáli Károly, Czakó László, Boros Mihály, Balogh Ádám, ifj. Lázár György:
The role of the glucocorticoid-dependent mechanism in the progression of sodium taurocholate-induced acute pancreatitis in the rat. *Pancreas* 2004; 29:75-82.

Paszt Attila, Éder Katalin, Szabolcs Annamária, Tiszlavicz László, Lázár György, Duda Ernő, Takács Tamás, ifj. Lázár György:
Effects of glucocorticoid agonist and antagonist on the pathogenesis of L-arginine-induced acute pancreatitis in rat. *Pancreas* 2008; 36:369-76.

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Importance of radiological and surgical treatment following necrotising pancreatitis.
European Congress of the International Hepato-Pancreato-Biliary Association (IHPBA), Budapest, 1999. *Dig. Surg*. 1999; 16:(Suppl. 1) 97.

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The role of glucocorticoid dependent mechanisms in the experimental acute pancreatitis
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1. INTRODUCTION

1.1. Pathophysiology of acute pancreatitis

The spectrum of acute pancreatitis (AP) ranges from a mild edematous disease to a severe necrotizing process which is usually accompanied by local or systemic complications. Although the exact mechanisms which trigger the inflammatory and necrotizing processes are not completely understood, it is generally accepted that autodigestion and an acute inflammatory response of the pancreas via the activation of proteases of the pancreas and activated leukocytes play important roles in the pathogenesis of AP. This amplified cytokine response is one of the most important pathophysiological factors in the development of complications, and especially in the progression of a limited local pancreatic inflammation into a potentially dangerous systemic inflammatory reaction termed multiple organ dysfunction syndrome. The incidence of AP has increased in the past two decades. The incidence of first-time attacks in the USA has increased from 33 to 44 per 100,000 adults, and at present AP accounts for more than 200,000 hospital admissions per year. AP is a relatively common disease in Europe, with a case incidence of 10 to 30 per 100,000 inhabitants. In 80% of the cases, the disease is mild, without significant morbidity or mortality. However, in 20% of the patients the disease is severe, with a stable frequency of mortality rate of around 25%-30%. The situation giving rise to AP determines its epidemiology. The most frequent cause is biliary stone migration. Although the etiological agents differ considerably, the ultimate outcome is relatively independent of the causative factors.

1.2. Scientific background

During the past twenty years, much evidence has accumulated which indicaties that proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), certain interleukins (IL-1 beta, IL-6, IL-8 and IL-10), prostaglandins, the arachidonic acid metabolism and many other biological active mediators play significant roles in the pathogenesis of AP, which can range from a mild illness to a lifethreatening condition. These mediators are primarily responsible for the distant organ complications (multiorgan failure) in severe AP.

The beneficial effect of glucocorticoid hormones in the treatment of human AP was originally reported by Stephenson *et al*. in the early 1950s. Since that time, the role of glucocorticoids has been investigated in various types of *in vivo* and *in vitro* experimental AP models. It has been reported to result paradoxically in the release of both anti-inflammatory (glucocorticoid hormones) and proinflammatory (macrophage migration inhibitory factor; MIF) mediators.

This counter-regulatory mechanism is essential for the provision of appropriate control of the ensuing inflammatory cascade.

The glucocorticoids are known to be involved in the regulation of cytokine production and the inflammatory processes. The corticosteroids inhibit the synthesis of TNF-α, IL-1, IL-6 and IL-8 and the function of nitric oxide synthase**,** and reduce complement activation and complement-induced leukocyte activation.

The proinflammatory cytokines activate the hypothalamic-pituitary-adrenal axis, stimulating pituitary adrenocorticotropin secretion and the resulting release of endogenous glucocorticoids, which are the most important endogenous modulators of inflammatory and immune responses. Recent studies have demonstrated the bidirectional communication between the neuroendocrine and immune systems, and this special feedback loop balances the intensity of the immune response and moderates the morbidity and mortality associated with the systemic inflammatory reaction.

Furthermore, glucocorticoids protect the acinar cells, stabilize the cell membrane and directly regulate amylase synthesis. Nevertheless, the effects of glucocorticoids on the clinical or experimental forms of AP have remained contradictory.

Low doses of glucocorticoids induce the secretion of MIF, a potent proinflammatory molecule which controls the synthesis of glucocorticoids and the action of inflammatory mediators (IL-1, IL-6, IL-8 and TNF). However, this regulatory role of MIF on the anti-inflammatory/immunosuppressive effects of glucocorticoid hormones has not been fully elucidated.

AIMS OF THE STUDY

Our primary goal was to create clinically relevant animal models suitable for investigations of the pathophysiology of AP and different treatment options.

We therefore set out to study the consequences of the administration of the exogenous glucocorticoid agonists dexamethasone (DEX), hydrocortisone (HYD) and methylprednisolone (MP) and the antagonist RU-38486 (RU) on on the local and systemic responses of AP, including the mortality, the severity, the levels of proinflammatory and inflammatory mediators, the local histopathological changes, and liver and lung injuries in experimental AP. Within this topic we investigated,

- the effects of glucocorticoid agonist and antagonist hormones on the mortality in AP;

- the effects of glucocorticoid agonist and antagonist hormones on the proinflammatory and inflammatory mediators in the plasma and in different target organs in AP;
- the effects of glucocorticoid agonist and antagonist hormones on the changes in pancreatitis markers in AP;
- the characterization of leukocyte infiltration by myeloperoxidase (MPO) in distant target organs the liver and lungs in AP;
- the role of adenosine triphosphate (ATP) level changes in distant target organs the liver and lungs in AP;
- the effects of glucocorticoid agonist and antagonist hormones on MIF level regulation;
- the role of glucocorticoid hormones in the regulation of the pancreatic nuclear transcription factor kappa B (NF-κB) activity;
- the effects of glucocorticoids on histological changes in the pancreas and distant organs the lungs and liver in experimental AP models.

2. MATERIALS AND METHODS

Animals

Male outbred Wistar rats (weighing 250-280 g) were used in all experiments.

2.1. Experimental protocols

2.1.1. Study I

The animals were allocated to one or other of eight groups. In group 1, AP was induced by the retrograde administration of 400 mg Na-taurocholate into the pancreatic duct (group AP). In groups 2, 3 and 4, the animals were given 4 mg/kg body weight (bw) dexamethasone (DEX) (Oradexon, Organon) (group APD), or 20 mg/kg bw hydrocortisone (HYD) (Solu-Cortef, Pharmacia&Upjohn) (group APH) or 5 mg/kg bw RU (Roussel-Uclaf, France) (group APRU) subcutaneously just prior to the induction of AP. RU was incorporated into multilamellar phospholipid liposomes prepared from phosphatidylcholine (Sigma Chem., U.S.A.) and cholesterol (Sigma Chem., U.S.A.) according to the method described earlier. In sham-operated groups 5-8, the animals underwent the same surgical interventions, but sterile saline was used as infusate into the pancreas (group Sham) or the animals received subcutaneously (sc.) the same doses of DEX (group ShamDEX), HYD (group ShamHYD), or RU (group ShamRU).

The animals were randomly assigned into one or other of three experimental series. In series 1 (n = 48), the mortality of AP was investigated. The animals were observed for 24 h, and the number of survivors was recorded. Series 2 (n = 178) was used for serum cytokine, amylase and pancreatic weight/body weight (pw/bw) determinations**.** Blood samples were obtained 2, 4, 8 and 24 h after AP induction. Tissue biopsies were taken for ATP and MPO measurements of the liver and lungs, and for histology of the pancreas, 24 h after AP induction. In the sham-operated series 3 (n = 60) blood samples were taken at 4 and 8 h, and tissue biopsies were taken at 24 h after surgery.

2.1.2. Study II

In the second study, the rats were randomly allocated into one or other of three groups. In the group AP, AP was induced by the intraperitoneal (ip.) administration of L-arginine (L-Arg) (2 x 250 mg/100 g bw). In groups APMP and APRU, the animals were given 30 mg/kg MP, (Organon) or 5 mg/kg RU (Roussel-Uclaf, France), respectively. The agonist and antagonist were administered sc. just prior to the induction of AP. In the control group (group C), the animals were given a saline injection sc. and were euthanized at 0, 8, 12 or 24 h without undergoing any other procedure. Each group contained at least 6 animals.

2.2. Surgical preparation

2.2.1. Experimental acute pancreatitis

In the first study, the surgical procedures were performed under Na-pentobarbital (40 mg/kg ip.) anesthesia. The abdomen was shaved, prepped, and draped in sterile fashion, and after a midline laparotomy, the pancreatic duct was cannulated at its entry into the duodenum, using a PE 50 catheter, fixed and connected to a pressure-controlled precision pump (IVAC, San Diego, CA). Prior to infusion, the common bile duct was occluded with a microvascular metal aneurysm clamp at the hilus of the liver, where the infusate entered the pancreas. 400 µl 3% Na-taurocholate (Sigma, U.S.A.) was perfused under standard parameters (pressure and volume) for 30 s. After the intraductal infusion, the bile duct clamp was removed, the cannula was withdrawn, and the abdomen was closed in two layers.

Int the second series, the experimental AP was induced by the ip. administration of L-Arg (2x250 mg/100 g bw).

2.2.2. Collection and analysis of plasma samples

Rats were laparotomized and sacrificed by abdominal aorta exsanguination under pentobarbital anesthesia 2, 4, 8, or 24 h in study I or 0, 8, 12 and 24 h following AP induction in study II. Blood samples were collected in heparinized tubes by exsanguinating the animals through the abdominal aorta. The blood was centrifuged at 3000 g for 10 min immediately after collection, and the plasma was separated through the use of sterile pipets and stored at -70 ºC until it was assayed.

2.3. Measurements

2.3.1. Survival analysis

In the first experiments, we investigated the survival rates 24 h after the induction of experimental AP by taurocholic acid. In the second series, survival analysis was not performed because in this dose the L-Arg-induced AP did not result in mortality.

2.3.2. Pancreatic weight / body weight (pw/bw) ratio determination

The pancreas was removed immediately after the blood collection, trimmed free of fat and lymph nodes, and weighed. The pw/bw ratio (mg/g) was calculated for each animal, to estimate the level of pancreatic edema.

2.3.3. Plasma amylase activity

Plasma amylase levels were measured by the amylase test of Phadebas *et al.* and are reported in standard units

2.3.4. Cytokine assays

2.3.4.1. Plasma IL-6 activity

IL-6 was bioassayed by using the murine hybridoma cell line B9, the proliferation of which is IL-6-dependent.

2.3.4.2. Macrophage migration inhibitory factor activity

Plasma MIF concentration was measured with a sandwich enzyme immunoassay kit (Chem. Int. Inc., USA) according to the manufacturer’s instructions.

2.3.5. Assay of inflammatory mediators in pancreatic tissue

2.3.5.1. Nuclear transcription factor kappa B analysis

The proinflammatory cytokines TNF and IL-1-beta are known to activate NF-κB. This fact was made use of to estimate the production of inflammatory mediators in tissue homogenates (where the reliability of other assays is limited).

Tissue biopsies were taken at 0, 8, 12 and 24 h following AP induction, trimmed free of fat and lymph nodes, weighed and frozen at -70 ºC until use. After mincing and ultrasonic homogenization, the debris was pelleted by low-speed centrifugation and the supernatant was used for the assay.

2.3.6. Reporter cell lines

Mouse L929 cells (5 x 10^5 cells on a 60-mm plate) were transformed with pNF-κB-luc4 and pSV-2/neo plasmids (coding for firefly luciferase under the control of 5 NF-κB-responsive elements and the *neo*^{r} gene controlled by the SV40 enhancer/promoter, respectively), using the DMIRIE-C cationic lipid transfection agent (GIBCO BRL). Selection on Geneticin G418 (400 mg/l) started 48 h later, the medium being refreshed twice weekly. Clones were isolated and tested for the intensity of their TNF-elicited NF-κB induction (50-100 U/ml recombinant TNF, 6-10 h of induction) and the most responsive clones were used in subsequent experiments.

RAW 264.7 cells (5 x 10^5/60-mm plate) subcultured on the previous day were transformed overnight with the above plasmids complexed with polyethylene-imine (jetPEI, Qbiogen, Illkirch, France).

2.3.6.1. Media

Both cell types were grown in MIX MEM (a 1:1 mixture of DMEM, Sigma Cat. no. D-7777 and F-12 HAM, Sigma Cat. No. N-4388) plus 10% fetal calf serum (FCS) (Sigma, Cat. no. F-4135). Transformation was carried out in OPTI MEM (GIBCO BRL).

2.3.6.2. Luciferase assay

One-day-old cultures (of both L929 and RAW 264.7 cells), grown on luminoplates (Corning-Costar Cat. no 3903) at 3 x 10^4 cells/well in MIX MEM 10% FCS, were exposed to tissue samples, TNF or LPS, respectively (in 100 μl of the above medium per well).

2.3.7. Analysis of liver and lung tissue samples

Simultaneously with the pancreas-removing processes, tissue samples were obtained from the liver and lungs and immediately processed, snap-frozen in liquid nitrogen for MPO and ATP assays, and stored at -37 ºC.

2.3.7.1. Myeloperoxidase activity

The tissue MPO activity, as a marker of tissue leukocyte infiltration, was measured in liver and lung biopsies by the method of Kuebler *et al*.

2.3.7.2. ATP measurement

Samples were taken from the liver and lungs with a Wollenberg forceps cooled in liquid nitrogen, and the tissue was stored at -70 ^{o}C. The ATP concentration was measured spectrophotometrically according to Lamprecht *et al*.

2.3.8. Histology

Tissue biopsies were taken for histology of the pancreas, lungs and liver 24 h after AP induction. The severity of the AP was determined by gross scoring of the macroscopic parameters of the pancreas pathology, including pancreatic edema, hemorrhage and fat necrosis.

The systemic damage involved in the pancreatitis was scored for 24 h in different target organs such as the liver and lungs.

2.3.9. Light microscopy

The hematoxylin and eosin stained slides were coded and read for the histological markers of tissue injury by one independent observer in a blinded fashion. The different special histological signs were graded semiquantitively in 8-10 consecutive high-power fields (x400). The score for each graded parameter was averaged and the total tissue damage was calculated by summing the averages.

2.3.10. Data analysis and statistical comparisons

Survival data were analyzed for statistical significance by the Fisher exact test. In the case of biochemical measurements for statistical analysis, a nonparametric method was used. Time-dependent differences from the baseline were assessed by Dunn’s method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks,

followed by Dunn's method for pairwise multiple comparison. P values <0.05 were considered statistically significant. Results are expressed as means ± S.E.M.

3. RESULTS

3.1. Study I

3.1.1. Survival analysis

In the non-treated AP group, the experimentally induced AP resulted in a 33% (4/12) survival rate, with a median survival of 10 h after an observation period of 24 h. However, the glucocorticoid agonists decreased the lethal effect of AP: in the groups APD and APH, the 24-h survival rates were 58% (7/12) and 75% (9/12), with a median survival of 17 and 19 h, respectively. In the group APRU, the resistance of the animals against AP was significantly decreased as compared with the groups APD and APH: the survival rate was only 17% (2/12). In the Sham group, the mortality was 0.

3.1.2. Pw/bw ratio

Pw/bw was significantly elevated in the group AP vs the normal condition ($p<0.05$). No difference between the groups AP and APRU was found at any time point. In the groups APD and APH, pw/bw was significantly diminished ($p<0.05$) vs the group AP. In the SHAM groups, the pw/bw ratio did not change significantly.

3.1.3. Plasma amylase activity

In the group AP, the amylase activity was significantly elevated at 2 h (13000 U/l ± 3000 U/l) and remained higher at 4, 8 and 24 h vs the basal level ($p<0.05$). In the glucocorticoid agonist-treated groups (groups APD and APH), the plasma amylase levels were significantly decreased as compared with the group AP at 2, 4 and 8 h ($p<0.05$). No significant alteration in amylase activity was found in the group APRU vs the group AP. In the SHAM groups, the glucocorticoid agonists and antagonists did not alter the basal amylase level significantly at any time point as compared with saline treatment.

3.1.4. Plasma IL-6 activity

In the group AP, the IL-6 level gradually increased, reaching its peak at 8 h (13500 U/l ± 3000 U/l). In the group APRU, the IL-6 value was significantly elevated (16000 U/l ± 3800 U/l) at 2h following AP induction as compared with the other groups ($p<0.05$). At 4, 8 and 24

h, its levels did not differ from those for the group AP. In the groups APD and APH, the IL-6 activity was significantly diminished ($p<0.05$) as compared with the group AP at 4, 8 and 24 h. The circulating IL-6 level did not change significantly in the SHAM group.

3.1.5. Liver and lung MPO activity

AP significantly elevated the leukocyte accumulation both in the liver and in the lungs ($p<0.05$) as compared with the SHAM group. RU treatment did not influence the MPO activities of the organs as compared with the group AP. DEX and HYD, however, significantly ($p<0.05$) attenuated the leukocyte accumulation in the liver 24 h following AP induction as compared with the AP group. In the glucocorticoid agonist and antagonist-treated SHAM groups, the MPO activity did not change significantly as compared with saline treatment.

3.1.6. Liver and lung ATP activity

AP alone resulted in significantly decreased ATP levels in both the liver and the lung tissue at the end of the observation period as compared with the Sham group ($p<0.05$). In the group APRU, the liver and lung ATP levels were similarly significantly ($p<0.05$) decreased vs the group Sham, but did not differ from that for the group AP. In the groups, APD and APH, the liver ATP levels were significantly ($p<0.05$) elevated as compared with the group AP. No significant differences were detected between the groups AP and APRU vs the groups APD and APH in the lung tissue ATP level. In the Sham groups (Sham, ShamDEX, ShamHYD and ShamRU), the ATP contents of the liver and lung tissue did not change significantly as compared with saline treatment.

3.1.7. Gross pathology

The scores were significantly improved in the groups APH and APD as compared with the group AP ($p<0.05$). In the AP and the groups APRU, macroscopic analysis revealed more severe pathological parameters than in the groups APH and APD.

3.1.8. Histology

Although the gross pathological and the microscopic findings following hematoxylin-eosin staining demonstrated appreciable histological changes in the pancreas, liver and lungs in the different groups, the differences in the investigated organs were not statistically significant.

3.2. Study II

3.2.1. Pw/bw ratio

The administration of L-Arg significantly increased the pw/bw ratio vs that in the group C ($p<0.05$). Glucocorticoid pretreatment tended to decrease the pw/bw ratio in the AP group, but this difference was not statistically significant.

3.2.2. Plasma amylase activity

In the AP and APRU groups , the amylase activities were significantly elevated at 12 h and remained higher at 24 h vs the basal level ($p<0.05$). In the glucocorticoid agonist-treated group (group APMP), the plasma amylase level was significantly decreased as compared with the groups AP and APRU at 12 and 24 h ($p<0.05$). No significant alteration in amylase activity was found in the group APRU vs the group AP.

3.2.3. Plasma IL-6 activity

In the group AP, the IL-6 level gradually increased, reaching its peak (1000 ± 200 U/l) at 8 h. In the group APRU, the IL-6 level was significantly elevated (750 ± 100 U/l) at 8 h and 12 h following AP induction as compared with the group APMP ($p<0.05$). In the group APMP, the IL-6 activity was significantly diminished ($p<0.05$) as compared with the group AP at 8 h. The circulating IL-6 level did not differ significantly between the groups AP and APM from 12 h up to the end of the observation period.

3.2.4. Plasma MIF level

AP induced increases in the baseline plasma MIF level at 8, 12 and 24 h. MP did not exert a significant effect on the plasma MIF level changes. RU treatment resulted in significantly higher MIF production at 8 and 12 h following L-Arg injection as compared with the groups APMP and AP.

3.2.5. Proinflammatory cytokines in the pancreas

In the early phase of AP, the level of pancreatic NF-κB activity changed in parallel in the MP- and RU-treated groups, and the difference between these groups was not statistically significant at 8 h following L-Arg injection.

MP treatment significantly decreased the level of NF-κB activity as compared with the RU-treated animals at 12 h following AP induction. Similarly, in the group APMP the pancreatic NF-κB concentration was significantly decreased as compared with the group AP at 24 h. RU

treatment resulted in a significantly higher level of pancreatic NF-κB activity at 12 h vs the groups AP and APMP. AP alone led to a significantly higher NF-κB activity as compared with the group C at 24 h.

3.2.6. Gross pathology

The administration of 2x250 mg/100 g bw L-Arg induced mild necrotizing AP.

The scores were significantly better in the group APMP as compared with the group AP ($p<0.05$). In the group APRU, the macroscopic analysis revealed more severe pathological parameters than in the group APMP.

3.2.7. Histology

However, at the end of the observation period, the extents of tissue damage in the pancreas and lungs were significantly less in the group APMP than in the groups AP and APRU.

4. DISCUSSION

AP is an inflammatory disorder of the pancreas with local and systemic manifestations, is a significant health problem. In spite of the improvements that have been achieved in intensive care and in surgical therapy, the mortality rate for necrotizing AP remains high. Activation of the inflammatory cascade, including different cytokines, results in microcirculatory disturbances and multiple organ failure. Among the most important aspects of the *in vivo* investigation of AP is the use of a relevant animal model which is able to mimic the clinical situation. Different experimental models of AP have been developed in order to seek an understanding of its pathophysiology, histological features and degree of severity, and the failure of distant organ systems.

In order to demonstrate the effects of glucocorticoids on AP we chose a severe model of AP. In study I, we applied the Na-taurocholate-induced AP model. This is an accepted and reproducible model of experimental AP, which produces the characteristic morphological and physiological changes of severe necrotizing type AP.

The biochemical and morphological data proved that Na-taurocholate administration did result in a severe necrotizing type of AP. In study II, we also chose a severe pancreatitis model. The L-Arg-induced noninvasive model is highly reproducible and standardizable, and produces selective, dose-dependent AP. With a low dose of L-Arg, the induced pancreatitis is not so severe and does not result in mortality. Increases in tissue corticosteroid levels during

an acute illness are important protective responses. There is now preliminary evidence to support the use of supplementary corticosteroids in patients with established septic shock, and especially those who display signs of functional hypoadrenalism.

However, the beneficial effects of exogenously administered corticosteroid hormones in experimentally induced AP are a matter of dispute**.** It is well known that the pituitary-adrenal axis, including glucocorticoids, plays a pivotal role in the mutual control mechanisms and communication between the neuro-endocrine and immune systems. During experimental AP, the extent of secretion of endogenous corticosterone is increased. Adrenalectomy is known to produce more severe AP and to elevate the serum IL-8 level.

Our results clearly demonstrate that glucocorticoid hormones attenuate the local and systemic effects of AP and prolong the survival of rats.

The injection of HYD during the development of AP was observed by Pescador *et al.* and by De Dios *et al.* to be harmful. In contrast, the protective effects of corticosteroids in AP have been reported by several authors. Furthermore, glucocorticoids have been found to attenuate the pancreas damage by protecting acinar cells during cerulein-induced AP, whereas the opposite was reported by Manso *et al.* in AP induced with a choline-deficient, ethionine-supplemented diet.

Our observations are in line with these findings, since the glucocorticoid agonists HYD, DEX and MP moderated the serum amylase and IL-6 activities and local inflammatory reactions. In contrast, the glucocorticoid antagonist RU amplified the inflammatory processes and resulted in an earlier IL-6 response to AP.

It has been established that the steroid receptor antagonist RU enhances the synthesis of TNF-α and sensitizes animals to the cytotoxic action of TNF-α. RU acts at the receptor level, and thus the increased TNF-α production seems to be mediated via the glucocorticoid receptors. As the most powerful inducer of IL-6 production is TNF-α, and glucocorticoids inhibit IL-6 synthesis directly, the earlier significant IL–6 production in the RU group can be explained.

One of the crucial points of these studies is the timing of steroid treatment during AP. Thcolakov *et al.* and Gloor *et al.* observed that treatment with a single prophylactic, low dose of exogenous steroid resulted in a decrease in the severity of AP. Repeated long-term HYD pretreatment has been demonstrated to worsen the inflammatory process in AP. Another controversial point is the dose of steroid treatment. High-dose corticosteroid treatment (mg/kg bw) increases the mortality rate, possibly due to secondary infection. On the other hand, low-dose (10 mg/kg bw) steroid treatment results in a more effective inhibition of inflammatory mediators (IL-8, TNF-α and arachidonic metabolisms), a better survival and a lower rate of

side-effects. The opposite was reported by Gloor *et al.*: they administered 10 mg/kg bw HYD 10 min following the induction of cerulein-induced AP, but this did not result in any protective effect. These controversial results might be explained by the differences in timing, the dose of glucocorticoid treatment and the type of experimental AP. We used twice as high a dose of HYD (20 mg/kg bw), and a much stronger glucocorticoid analog, DEX, in a dose of 4 mg/kg bw. In spite of the differences in biological half-life, the two steroid analogs achieved the same result. These findings suggest that even the glucocorticoid compound with weaker anti-inflammatory potency (i.e. HYD) could adequately attenuate the inflammatory mediator response in this setting.

Recent studies have led to the rediscovery of a pituitary mediator that appears to act as the counter-regulatory hormone for corticoid action within the immune system. The protein historically known as MIF was identified more than 40 years ago as a product of antigen-sensitized lymphocytes that could inhibit the random movement or migration of cultured mononuclear cells *in vitro*.

Low concentrations of glucocorticoids directly induce MIF release from macrophages and T cells. This finding was initially surprising because it was generally considered that glucocorticoids inhibit the release of proinflammatory mediators. The glucocorticoid-induced secretion of MIF is tightly and dose-dependently regulated. At high anti-inflammatory glucocorticoid concentrations ($>10^{-8}$ M), the secretion of MIF is known to be completely shut off.

It is of importance that MIF circulates normally in the blood. The basal MIF levels have been estimated by ELISA techniques to range from 2 to 4 ng/ml amounts that demonstrate glucocorticoid counter-regulatory activity *in vitro*. These data suggest that the physiological state of the glucocorticoid-MIF system is one of an active balance between pro- and anti-inflammatory mechanisms.

MIF production seems to be upregulated in other cases of pancreatitis too, and its presence can be regarded as an early marker of pancreatic necrosis in AP.

We demonstrated that the induction of AP by the injection of L-Arg resulted in a marked increase in the MIF concentration in the plasma. There was a continuous tendency for the administration of high doses of MP to elevate the plasma MIF level, but this effect was not statistically significant. Our finding had previously been suggested by recent studies on the effects of glucocorticoids on MIF production in different acute inflammatory processes. Nagasaki *et al.* reported that MP did not increase, but rather tended to decrease the serum MIF level. Similarly, in experimental rat models Bruhn *et al*. observed that the administration of

DEX did not enhance MIF production in sepsis. Our results are in accord with the findings of these authors, who concluded that exogenously administered glucocorticoid treatment downregulated the level of cell-associated MIF and MIF release by various activators. The fact that MP did not further increase the production of MIF in our experiments suggested that the degree of glucocorticoid-induced MIF production was already maximal in the AP animals because of the high level of endogenous glucocorticoids. On the other hand, one of the most interesting observations in the second phase of the study was that RU treatment resulted in significant elevations of the plasma level of MIF 8 and 12 h after AP induction. However, there is no clear-cut explanation for this fact. Very little is known about the regulation of the MIF gene; some data suggest that NF-κB is involved.

Glucocorticoid hormones diminish the production of inflammatory cytokines via the inhibition of cytoplasmic transcriptional factors such as NF-κB. The glucocorticoids activate glucocorticoid receptors that may then bind to activated NF-κB and activator protein-1, preventing binding to κB sites on genes that have a role in inflammatory processes.

Our important major finding is that MIF counteracts the glucocorticoid-mediated inhibition of NF-κB in L-Arg-induced experimental AP in rats. Our results suggest that NF-κB activation in L-Arge-induced AP occurs at a later stage as compared with other models. A significant NF-κB elevation was detected 1 h after the induction of AP with Na-taurocholate.

We found that RU treatment led to in significantly higher NF-κB activity 12 h after pancreatitis induction, which can be explained in that RU treatment results in significantly higher MIF production at this time. It was clear that the short-term pretreatment with a high dose of glucocorticoid caused a significant lowering of the NF-κB activity in the later phase of AP.

Many inflammatory components are regulated by NF-κB, including inflammatory cytokines, acute-phase proteins, etc. Glucocorticoid hormones are known to diminish the production of inflammatory cytokines via the inhibition of cytoplasmic transcriptional factors such as NF-κB. Glucocorticoids prevent NF-κB activation in part by increasing the expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), which keeps NF-κB bound in the cytosol and thereby prevents the gene expression of proinflammatory mediators. The mechanism for the counter-regulatory action of MIF involves the prevention of glucocorticoid-induced increases in cytosolic IκBα. As a consequence, NF-κB can translocate to the nucleus and activate transcription, even in the presence of normal physiological concentrations of HYD. MIF could activate an intracellular pathway, leading to IκBα kinase activation and consequences of IκBα degradation, in this way overriding the

glucocorticoid-induced inhibition of NF-κB activation. Moreover, glucocorticoid increases the expression of IκBα, preventing NF-κB translocation to the nucleus and the activation of transcription.

We demonstrated that experimental animals respond to AP with a significantly higher degree of tissue leukocyte infiltration, as evidenced by the increased MPO activity in the liver and lungs. It is noteworthy to note that in experimental AP the glucocorticoid agonists attenuated the MPO activity. This finding underlines the importance of the action of glucocorticoid in the control of inflammatory mediator-induced remote organ damage.

Previous nuclear magnetic resonance studies have revealed the depletion of high-energy phosphates in pancreatic cells in AP, which seems to occur as a result of pancreatic cell death. Halangk *et al.* reported a correlation between the degree of depletion of high-energy phosphates and the severity of AP. In this process, the main source of the enhanced permeability of the mitochondrial inner membrane is the inhibition of complex I of the respiratory chain. The hepatocellular dysfunction and ATP depletion in AP are also well known, but little attention has been paid to the relationship of steroid treatment and the high-energy phosphate level in inflammatory processes of the pancreas. In our study in animals with AP, a significant ATP depletion was demonstrated in remote organs too. The observation that ATP depletion develops in the lungs provides a possible explanation for the pulmonary dysfunction seen in AP. It is also possible that other systemic complications, such as a decreased myocardial contractility or renal failure, are indirectly related to the mitochondrial dysfunction and ATP depletion caused by circulating cytokines and other toxic components. Therefore, our results may suggest a novel explanation for the multiple organ dysfunction and increased morbidity in AP. On the other hand, low-dose steroid treatment was effective in our study, glucocorticoid agonists significantly elevating the liver ATP content in experimental AP.

In our series in the Na-taurocholate-induced AP model, the histological changes in target organs were more characteristic than in the L-Arg-induced series. We proved the protective effect of glucocorticoid analog treatment on tissue damage. Moreover, at the end of the observation period, the extents of tissue damage in the pancreas and lungs were significantly less in the glucocorticoid-treated (APMP) groups than in AP and after Ru treatment.

In conclusion, our study highlights the fact that glucocorticoid hormones play an important role in maintaining the resistance of the animals against the lethal effects of experimentally induced AP. Furthermore, the exogenously administered glucocorticoid agonists greatly moderate the local and systemic inflammatory consequences of experimental AP.

5. SUMMARY AND NEW FINDINGS

The histological and biochemical findings proved that both Na-taurocholate and L-Arg induced a severe type of AP. These two reproducible and standardized *in vivo* experimental models provide useful protocols which furnished valuable information on the functional, morphological and biochemical changes during the initiation phase of AP, and on the pathophysiological and morphological consequences of AP in response to different treatments.

Our results clearly demonstrated that glucocorticoids critically mitigate the progression of the inflammatory reaction during the early phase of experimental AP.

Exogenously administered short-term pretreatment with high-dose glucocorticoid hormones attenuated the local and systemic effects of AP, decreased the serum amylase and IL-6 activities and local inflammatory reaction, and prolonged the survival of the animals.

Our study has proved that experimental animals respond to AP with a significantly higher degree of tissue leukocyte infiltration, as evidenced by the increased MPO activity in the liver and lungs. It must be noted that in experimental AP the glucocorticoid agonists attenuated the MPO activity.

Our study demonstrated a significant ATP depletion in the liver and the lungs of animals with AP. The observation of an ATP depletion in the lungs provides a possible explanation for the pulmonary dysfunction seen in AP. Steroid treatment was effective in our study, glucocorticoid agonists significantly elevating the liver ATP content in experimental AP. Furthermore, inhibition of the endogenous corticosteroid action by RU treatment also resulted in a pronounced ATP depletion in remote organs, this process being inhibited by glucocorticoid agonists.

The induction of AP by the injection of L-Arg led to a marked increase in the MIF concentration in the plasma. There was a continuous tendency for the administration of high doses of MP to enhance the plasma MIF level, but this effect was not statistically significant. On the other hand, treatment with the antiglucocorticoid RU gave rise to significant elevations of the plasma level of MIF after AP induction.

The other important major finding was that MIF counteracted the glucocorticoid-mediated inhibition of NF-κB in L-Arg-induced experimental AP in rats. Our results suggested that NF-κB activation in L-Arg-induced AP occurs at a later stage as compared with other models.

We clearly demonstrated that short-term pretreatment with a glucocorticoid agonist resulted in a significant reduction of NF-κB activity in the later phase of AP.

In conclusion, our study clearly revealed that glucocorticoid agonist treatment promotes the resistance of animals against the lethal effects, and provides considerable protection against the systemic toxicity of inflammatory cytokines of experimentally induced AP.

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