The role of Kupffer cells in mediating the microcirculatory and biochemical consequences of endotoxemia and obstructive jaundice in rats

Ph.D. Thesis

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1. INTRODUCTION

1.1. Septic complications of obstructive jaundice

Patients with obstructive jaundice undergoing surgical procedures have a significant risk of complications and death. Gram-negative sepsis constitutes the bulk of the morbidity and mortality, although renal dysfunctions, coagulopathy, gastrointestinal hemorrhage and impaired wound healing are also well recognized.

Clinical and experimental studies have suggested several etiological factors for these complications, including hypotension, an impaired nutritional status, an impaired immune function and the presence of potential toxic substances in the circulation, such as bilirubin and bile acids. However, in recent years, there has been an increasing recognition of the role of circulating endotoxins in the development of complications in obstructive jaundice. Many authors, using a variety of clinical and biochemical parameters, have attempted to identify those jaundiced patients most at risk; neverless, there has been no universally accepted theory for the pathophysiology of the complications seen in biliary obstruction. In surgical practice, biliary obstruction is often combined with septic complications. A microcirculatory dysfunction and leukocyte activation are interdependent components of acute liver injuries, including lipopolysaccharide (LPS)-induced hepatic damage. Intrahepatic leukocyte activation and microvascular perfusion failure are also involved in the pathology of biliary obstruction. The precise mechanisms that participate in the susceptibility of jaundiced patients to sepsis, however, are still unclear and the relative impact of an increased LPS load on a cholestatic microcirculatory dysfunction is as yet undefined. Gram-negative sepsis and its sequelae occur frequently in patients with extrahapatic obstructive jaundice following invasive diagnostic or therapeutic procedures. Normally, small quantities of endotoxin in the portal circulation are cleared by Kupffer cells (KCs).

We therefore applied clinically relevant models of obstructive jaundice in combination with endotoxemia in rats, in order to test whether the microcirculatory and biochemical consequences of bile duct ligation (BDL) are deteriorated by an additional LPS challenge.

1.2. The role of Kupffer cells in different inflammatory processes

The liver KCs comprise the largest macrophage population in the human organism. KCs, resident liver macrophages, constitute 80-90 per cent of the fixed-tissue mononuclear cell population. They are positioned at the interface between the portal and systemic circulations and are responsible for sequestering pathogenic substances, including bacteria and toxins, from the portal circulation. The KCs of the hepatic reticuloendothelial system (RES) synthesize and secrete various bioactive compounds, including reactive oxygen and nitrogen radicals, eicosanoids and peptide mediators,

forming the first line of defense against microorganisms entering the portal circulation. KC activation and the subsequent secretion of inflammatory mediators are fundamental for an effective immune response. Persistently high or overwhelming activation may result in an uncontrolled initiation of the proinflammatory cascade, leading to the systemic inflammatory response syndrome and potentially to the multiple organ dysfunction syndrome. Mediating an appropriate immune response, KC activation is likely to play protective roles in the acute phase of hepatic pathologies. However, in response to an ongoing bacterial or endotoxin challenge, KC activation may increase the severity of the liver dysfunction. In LPS-induced sepsis, KCs eliminate LPS and other agents primarily from the portal blood, protecting the hepatocytes from exposure to high levels of LPS and other oxidative stresses. However, it is believed that LPS-stimulated KCs produce tumor necrosis factor-alpha (TNF-α), resulting in hepatocyte and endothelial damage, indicating that activation of the KCs is responsible for liver damage. In experimental biliary obstruction models, an enhanced susceptibility of jaundiced animals to endotoxemia has been demonstrated, which was critically linked to the activation of KCs. The KC functions are altered after biliary obstruction, the KC-dependent immune modulation then possibly leading to divergent outcomes. Defects in crucial elements of the function of the RES after cholestasis lead to hypersensitivity to LPS, with a high rate of septic complications in the long run. Most of the above reactions and also the production of the proinflammatory cytokines were enhanced when obstructive jaundice was followed by a second hit of LPS, and biliary obstruction exacerbates the hepatic microvascular inflammatory response to endotoxin.

Macrophage blockade has the theoretical advantage of abrogating inflammatory responses at an earlier stage of sepsis. Rare earth metal salts, including gadolinium chloride ($GdCl_3$) depress the RES activity and selectively interfere with the function of the KCs. $GdCl_3$ inhibits the secretion of biologically active substances from the liver KCs, and the liver-damaging effects of hepatotoxins, ischemia reperfusion and the develpoment of septic shock. However, it has been demonstrated that attenuation of the KC activity with $GdCl_3$ might decrease the LPS-induced lethality and morbidity in obstructive jaundice. In the present studies, we also applied this compound as a 24-h pretreatment in the different models (during BDL with or without endotoxemia). During our examinations, we also wanted to test the possible side-effects of this treatment. We hypothesized that a change in the level of activation of the KCs would be a significant determinant factor in the pathomechanism of the inflammatory consequences of obstructive jaundice.

1.3. The role of leukocytes in various inflammatory processes in the liver

Endotoxemia/sepsis induces an inflammatory response, with the leukocytes primarily contributing to hepatocellular injury by the release of a variety of mediators. Although, in turn,

necessary for vital host defense mechanisms, leukocytes can considerably aggravate tissue injury. Previously, the intrasinusoidal sequestration of leukocytes with transmigration into the tissues has been identified as a critical step of endotoxin-induced liver injury. After transmigration, the neutrophils attack the parenchymal cells and cause severe liver cell necrosis. Within this process, apoptotic hepatocytes have been shown to function as chemotactic signals, triggering leukocyte transmigration and thereby sustaining the cell-dependent inflammatory response.

Investigations utilizing intravital microscopy (IVM) have demonstrated that the recruitment of inflammatory cells into the perivascular tissue involves a complex cascade mechanism. The adhesion process consists of several steps, beginning with the rolling of polymorphonuclear leukocytes (PMNs) on the endothelial surface of the postcapillary venules until they have slowed down to such a degree that they stick to the endothelium. At this point, the leukocytes are sequestered from the main vascular flow, and firm adherence to the endothelial cells may follow. Subsequently, the leukocytes pass an intercellular junction between the endothelial cells and reach the abluminal side. Three families of leukocyte-endothelial adhesion molecules have been identified: the selectins, the immunoglobulin gene superfamily, and the integrins. The selectin family comprises three proteins, designated by the prefixes L (leukocyte), P (platelet) and E (endothelial). This is a class of cell adhesion molecules which mediate leukocyte rolling on the endothelium. P-Selectin (CD62P), which is stored in the Weibel-Palade bodies of the endothelial cells, is rapidly mobilized to the plasma membrane in response to proinflammatory mediators such as thrombin or histamine. L-Selectin (CD62L) is expressed on most types of leukocytes and is shed from the cell membrane by proteolytic cleavage after cellular activation. E-Selectin (CD62E), which is not expressed on the endothelial cell membrane under basal conditions, is synthesized after stimulation by inflammatory mediators such as TNF-α and endotoxin. After the leukocyte has been arrested, integrins are activated by chemokines, chemoattractants and cytokines. During the transmigration process, a vascular dysfunction may occur due to the inappropriate release of oxidants, proteases and other potent mediators of the activated leukocytes. We used IVM to observe the microcirculatory consequences of the above challenges directly in the liver. This method provided a possibility for the nearly online quantification of hepatic perfusion changes and of the primary and secondary PMN-endothelial interactions. Furthermore, this tool enabled us to quantify the hepatic activation of KCs.

1.4. The role of free radicals in sepsis and organ injury

The mechanisms involved in shock and organ injury induced in septic shock are multifactorial. Diverse molecular mechanisms of inflammation and cellular damage have been implicated in the pathogenesis of septic shock and multiple organ failure, including those related to the overt generation

of cytokines, eicosanoids and reactive oxygen species, such as nitric oxide, superoxide anion or peroxynitrite. Under normal physiological conditions, the majority of reactive oxygen species are formed during cellular respiration and by activated phagocytic cells, including PMNs and KCs, and a homeostatic balance exists between the formation of reactive oxidizing/oxygen species and their removal by endogenous antioxidant scavenging compounds. Oxidative stress occurs when this balance is disrupted by the excessive production of oxygen and nitrogen-derived free radicals, and/or by inadequate anti-oxidative defense mechanisms. As a result, oxidation of DNA and proteins may take place, along with membrane damage because of lipid peroxidation (LPO), leading to alterations in membrane permeability, modification of protein structure and functional changes.

The most efficient enzymatic antioxidants involve superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase. SOD is a highly effective intracellular enzymatic antioxidant which catalyzes the dismutation of $O_2^{\bullet-}$ to oxygen and to the less-reactive species hydrogen peroxide by catalyzing the dismutation of the superoxide anion to oxygen and water. There are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD. CAT is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert 6 million molecules of hydrogen peroxide to water and oxygen each minute.

1.5. Heme oxygenase and metallothioneins as special components of the antioxidant defense system

Tissue damage resulting from endotoxemia or obstructive jaundice also leads to the activation of endogenous protective mechanisms. Among the upregulated proteins, the expression of the microsomal enzyme heme oxygenase (HO) is triggered by both endotoxemia and biliary obstruction. HO plays role a in heme degradation, and also produces carbon monoxide, a vasoactive dilalator agent with important free radical scavenger properties. The fact that stress reactions, including endotoxemia, are associated with HO upregulation in the KCs, suggests that these self-defensive anti-inflammatory reactions are also initiated in this important proinflammatory cellular component of the liver.

There are also other, albeit not enzymatic proteins in the liver with metal-binding and considerable anti-inflammatory properties. Metallothioneins (MTs) are low molecular weight, non-enzymatic proteins that play a homeostatic role in the control and detoxification of the heavy metals. MTs are constitutively expressed in the liver and are overexpressed in endotoxemia. As one-third of its amino-acid residues are cysteines, MT provides a neutrophilic sink binding electrophiles. MTs may ameliorate the effects of oxidative stress by scavenging free radicals and even by preventing their formation. The purpose of the second part of the study was to examine the effects of endotoxemia with

or without biliary obstruction on hepatocellular damage, on the endogenous antioxidant mechanisms, and on the HO and MT expression changes, by using molecular biological methods. Furthermore, we wanted to elucidate the effects of the KC blockade with $GdCl_3$ on these parameters.

2. AIMS

The aims of the present study were:

1. To evaluate the hepatic inflammatory consequences of 3-day obstructive jaundice in the presence and absence of acute endotoxemia:

- By examining the early inflammatory complications of obstructive jaundice with *in vivo* microscopic methods. With this aim, we performed experiments to characterize the inflammatory microvascular alterations (hepatic microvascular perfusion and leukocyte-endothelial interactions).
- By quantification of the accumulation of neutrophilic leukocytes by means of hepatic myeloperoxidase (MPO) activity measurements.
- By assessing the expression changes in proinflammatory cytokine (interleukin-6 (IL-6) and TNF-α) release.
- By characterizing the isoform-specific HO and MT gene induction changes.
- By examining the functional and structural indices of liver injury, oxidative hepatic injury (DNA damage and lipid peroxidation) and histological liver injury.
- By assessing the changes in activity of the different components of the endogenous anti-oxidant defense system (CAT and Mn- and Cu/Zn-SOD) during the above challenges.

2. To examine the activation of KCs by using an IVM approach and to assess their role in the above changes by inhibition of the KC function with 24-h $GdCl_3$ pretreatment.

3. MATERIALS AND METHODS

3.1. Experimental protocol and experimental groups

Male outbred Wistar rats from the University of Szeged (weighing 250-300 g) were maintained on standard laboratory diet and tap water *ad libitum* and housed in an environmentally controlled room under a 12-h light - 12-h dark cycle.

Two major experimental series were performed. In the first series, the animals were allocated randomly to one or other of four groups. In the first group, endotoxemia was elicited by injecting a low dose (1 mg/kg bw) of LPS (*E. coli* 026:B6 LPS B. Difco; Laboratories, Detroit, MI, USA) i.v.

through the tail vein, 2 h before the IVM measurements (LPS group, $n=5$). In another group, extrahepatic biliary obstruction was induced by BDL 3 days prior to the measurements (BDL group, $n=5$). Briefly, a short midline abdominal incision was made and the common bile duct was ligated with a 4-0 silk suture under light oxygen-ether anesthesia. The abdomen was then closed in two layers and the animals were allowed to recover. In a third group, these challenges were combined (BDL + LPS group). The data were compared with those on sham-operated animals (Sham group, $n=5$). The animals in all groups received 1 ml/kg saline i.v. through the tail vein 24 h before the examinations.

After characterization of the consequences of the above major challenges, additional experimental groups were used to examine the effects of KC blockade (Series 2). In this series, the rats were pretreated with $GdCl_3$ (10 mg/kg bw i.v. through the tail vein; Prolabo, Paris, France) 24 h before the IVM examinations; the animals were then challenged with the sham operation (Sham + $GdCl_3$ group, $n=5$), with BDL (BDL + $GdCl_3$ group, $n=5$), with LPS (LPS + $GdCl_3$ group, $n=5$) or with their combination (BDL + LPS + $GdCl_3$ group, $n=5$). At the end of the experiments, blood samples for proinflammatory serum cytokine (TNF-α and IL-6) assessment and liver biopsies for biochemical and histological evaluations (see later) were taken.

3.2. Microcirculatory parameters
3.2.1. Intravital fluorescence microscopy of the liver

For the IVM examinations, animals were anesthetized with sodium pentobarbital (45 mg/kg i.p.), placed in the supine position on a heating pad (the body temperature was kept at 36-37 °C) and tracheotomized to facilitate spontaneous respiration. Polyethylene catheters placed in the right carotid artery and jugular vein allowed for the monitoring of the systemic hemodynamics (mean arterial pressure and heart rate), blood sampling and injections of fluorescent dyes for IVM. Following a transverse subcostal laparotomy, the left liver lobe was exteriorized, placed on a specially designed pedestal, and covered with a glass slide and a heating pad, turned on its left side, providing a suitable horizontal plane of the liver lobe for intravital microscopic examinations. The hepatic microcirculation was analyzed by means of an epi-illumination technique, using a fluorescence videomicroscope (Zeiss Axiotech Vario 100HD supplied with a 100 W HBO mercury lamp and an Acroplan 20x water immersion objective) with a blue (450-490/>515 nm) and a green (525-555/>580 nm) filter system. The microscopic images were recorded by a charge-coupled device video camera (AVT HORN-BC 12) attached to a video system (Panasonic AG-MD 830). The contrast enhancement was achieved by injecting sodium fluorescein (75 µg/kg i.v., Sigma, St. Louis, MO, USA). Leukocytes were stained *in vivo* by means of rhodamine-6G (0.2%, 0.1 ml i.v., Sigma, St. Louis, MO, USA). For the IVM analysis of KC phagocytic activity, plain fluorescent latex particles (diameter 1.0 µm; Polysciences

Inc., Warrington, PA, USA) were injected i.v. (3×10^8/ml/kg sterile isotonic saline as a bolus injection). At the end of the 30-min observation period, plasma samples (for IL-6 and TNF measurements) and liver biopsies (for biochemical and histological evaluations) were taken and the animals were killed with an overdose of pentobarbital.

3.2.2. Video analysis

Capillary perfusion, leukocyte-endothelial interactions and KC activity were quantified off-line, using computer-assisted analysis of the video recordings (IVM Software, Pictron Ltd., Budapest, Hungary). The *microcirculatory perfusion failure* was quantified by calculating the ratio of perfused and non-perfused capillaries within 10 acini per animal. *Leukocyte-endothelial interactions* were described by calculating the number of sticking leukocytes within 5 central venules per animal. A leukocyte was defined as firmly adherent if it remained stationary for at least 30 s. The number of leukocytes sticking to the endothelial surface (mm^2) was calculated from the diameter and length of the vessel segment, assuming cylindrical geometry. *The phagocytic function of the hepatic macrophages* was assessed by measuring the phagocytosis of fluorescent 1.0 µm latex particles by individual cells. The number of macrophages was measured via the number of phagocyted latex microspheres within 10 acini in each animal 20 min after injections of the microbeads.

3.3. Biochemical parameters

3.3.1. Myeloperoxidase activity

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

3.3.2. Cytokine assays

TNF-α cytotoxicity was measured by standard procedures, using mouse WC-1 tumor cells in the presence of 1 µg/ml actinomycin-D at 37 °C by the method of Aggarwal *et al..*

IL-6 was measured via the degree of proliferation of a murine hybridoma cell line (B9), which grows only in the presence of IL-6.

3.3.3. RNA extraction, reverse transcription and PCR amplification

For molecular biological examinations, the liver tissues were removed, frozen immediately in liquid nitrogen and stored at –80 °C. Approximately 100 mg of frozen tissue was homogenized in RNAzol B reagent (Tel-Test, Inc., Friendswood, TX, USA) and the total RNA was prepared according to the procedure suggested by the manufacturer. The total RNA was routinely treated with 100 U of RNAse-free DNAse I to avoid any DNA contamination. To quantify MT and HO-specific mRNAs, an RT-PCR-based strategy was employed. First-strand cDNA was synthetized by using 5 µg total RNA as template. The RNA was denatured at 90 °C, and mixed with 200 pmol of each dNTP (Sigma, St.

Louis, MO, USA), 200 U of M-MuLV reverse transcriptase (Sigma, St. Louis, MO, USA) and 500 pmol of random hexamer primer. The reaction mixture was incubated for 10 min at 37 °C, followed by 1 h at 42 °C. The reaction was stopped by heating at 65 °C for 5 min. 2 μl of reverse transcription product was added to 48 μl of PCR reaction mixture containing 250 μmol of each dNTP, 1x Sigma PCR buffer/MgCl₂, 5 U of Taq polymerase (Sigma St. Louis, MO, USA) and 50 pmol of primers specific to the MT-1, MT-2 and HO-1 and HO-2 isoforms and the β-actin gene. Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The number of amplification cycles during which PCR product formation was limited by the template concentration was determined in pilot experiments: for β-actin 25, and for MTs and HOs 30 cycles were used. The amplified products were electrophoresed on 2% agarose (Sigma, St. Louis, MO, USA) gel.

3.3.4. Primers and measurement of metallothionein and heme oxygenase mRNA levels

For the amplification of rat MT and HO mRNAs, isoform-specific primers were designed on the basis of the databank entries M11794 and AY341880 for the MT-1/2 isoforms, and NM_012580 and NM_024387 for the HO-1/2 isoforms. For normalization of the amounts of MT and HO mRNAs, the β-actin mRNA level was used as internal standard. The sequences of the primers β-actin-3 and 4 were derived from GeneBank entry M24113. Images of ethidium bromide-stained agarose gels were digitalized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot™ Pro Gel Analysis Software (UVP Inc., San Gabriel, CA, USA). The relative levels of MT and HO mRNAs are expressed as the ratio MT/β-actin or HO/β-actin. For each experimental treatment, 5 animals were used to prepare RNA. RT-PCR reactions for each animal were performed in triplicate to increase the reliability of the measurements.

3.3.5. Catalase activity

CAT activity was determined spectrophotometrically at 240 nm by the method of Beers *et al*.

3.3.6. Superoxide dismutase activity

SOD activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation. Mn-SOD activity was measured by the autoxidation method in the presence of 5×10^{-3} M KCN. Cu/Zn-SOD activity was calculated by deduction of the Mn-SOD activity from the total SOD activity.

3.3.7. Lipid peroxidation

LPO was estimated from the formation of thiobarbituric acid-reactive substances, determined by using a modification of a method described by Serbinova *et al*.

3.3.8. DNA single-strand breaks

The alkaline fluorescence analysis of DNA unwinding was used to determine single-strand DNA breaks. DNA samples were prepared from the livers of control and treated animals by using the salting-out method of Miller *et al*.

3.4. Histology

Tissue specimens from livers were fixed in 10% buffered formalin and embedded in paraffin. Representative tissue sections were cut (6 µm) from paraffin blocks and stained with hematoxylin and eosin for light microscopy. Histological analysis and the scoring of damage were performed in coded sections by a skilled histologist. Portal, periportal and intralobular evaluations were used to quantify the degree of damage, using a 0-5 grade damage scoring system.

3.5. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Changes in variables within and between groups were analyzed by two-way ANOVA followed by the Holm-Sidak test. *P* values < 0.05 were considered statistically significant.

4. RESULTS

4.1. Microcirculatory changes

In response to BDL, significantly lower rates of perfused capillaries were found (approximately 63%) than in the sham-operated animals (approximately 89%). Furthermore, the perfusion failure caused by BDL was greatly potentiated by the LPS challenge, whereas the rate of the perfused capillaries did not reach 50%. A slightly ameliorated hepatic capillary perfusion failure in response to KC blockade by $GdCl_3$ pretreatment was found in the BDL + LPS group.

Significantly higher numbers of firmly adherent leukocytes in the central venules were found after LPS (approximately 5-fold) and BDL + LPS (approximately 6-fold) in comparison with those in the sham-operated group. These changes were completely abolished by $GdCl_3$ in the LPS group and significantly ameliorated in the animals challenged with BDL + LPS.

The phagocytic activity of the KCs was estimated via the number of phagocytosed latex microspheres. These values were approximately 3-fold higher in the BDL and LPS-treated animals, and approximately 5-fold higher in the combined group, in comparison with the shams. These changes were reduced by $GdCl_3$ treatment in each of the above groups.

4.2. Biochemical changes

As evidenced by the MPO measurements, obstructive jaundice caused a moderate, while LPS induced a marked leukocyte accumulation in the liver, which was greatly augmented when these challenges were combined. In this latter group, $GdCl_3$ significantly attenuated PMN deposition in the liver.

The serum IL-6 and TNF-α levels were nearly undetectable in the sham-operated and BDL-challenged groups, but were elevated and exhibited similar tendencies of change after BDL + LPS. Moreover, both the IL-6 and TNF-α levels were approximately 5 times higher after BDL + LPS than after LPS alone. $GdCl_3$ pretreatment inhibited only the TNF-α release in the animals challenged with LPS + BDL.

HO-1 appeared to be highly inducible by all of the stimuli employed. Specifically, BDL caused an approximately 4-fold elevation, whereas LPS alone led to a nearly 10-fold elevation in this parameter. When LPS was combined with BDL, a similar extent of HO-1 induction was observed to that seen with LPS alone. In the sham-operated animals treated with $GdCl_3$, no changes in this parameter were detected, but the LPS or BDL + LPS-induced increases in HO-1 were significantly reduced following this intervention. HO-2 induction, however, led to a significant elevation (approximately 2-fold) only in the LPS-treated groups, where the alleviating effect of $GdCl_3$ was also evident.

No increases in the expression of the MT-1 gene were found in response to the different stimuli, but an enhanced MT-2 gene expression (approximately 3.5-fold) could be demonstrated after LPS alone or when LPS was combined with BDL. No alterations in this parameter were observed in the vehicle-treated animals subjected to 3-day BDL. Significant and similar degrees of increase in MT-2 induction were found in all $GdCl_3$-treated groups, irrespective of the different challenges applied.

As compared with the sham-operated animals, BDL and BDL + LPS caused severe (>50%) reductions in CAT activity, whereas LPS alone gave rise to an approximately 30% decrease in this parameter $GdCl_3$ itself resulted in some reduction (by ~10%) in CAT activity. However, when $GdCl_3$ was applied in the presence of endotoxemia (or endotoxemia combined with BDL), it caused a partial restoration in CAT levels.

An approximately 2-fold increase in Mn-SOD activity was found in all of the challenged groups, but the Cu/Zn-SOD activity did not change significantly; $GdCl_3$ did not affect these alterations.

A moderately enhanced and comparable degree of DNA damage (approximately 4-fold) was observed in all challenged groups with or without KC blockade elicited by the heavy metal salt $GdCl_3$,

and a similar degree of DNA damage was also evidenced in response to this treatment alone. A lower level of DNA breakage was observed only in the animals challenged with BDL in the presence of $GdCl_3$ treatment.

The MDA content in the liver was increased significantly (4-fold) only in the most severe condition, when endotoxemia was combined with BDL. $GdCl_3$ enhanced the LPO (2-3-fold) in all groups (including the sham-operated animals) and the degree of LPO again appeared to be independent of the type and severity of the challenge.

4.3. Histological damage in the liver

Histological evaluation was performed in the portal, periportal and intralobular regions of the liver. The sham operation or LPS treatment did not induce considerable structural alterations in any of the regions examined. The present grading system indicated that BDL and BDL + LPS caused the most marked damage in the portal region, but structural damage was also evident in the periportal and intralobular regions. Interestingly, the above histological alterations were similar in the BDL and BDL + LPS groups. These changes were not influenced by the KC blockade in the portal and intralobular regions, but were completely abolished in the periportal area.

5. DISCUSSION

The survival rate of critically ill patients with obstructive jaundice has not improved in recent decades and septic complications are still the leading causes of mortality in this condition. In line with this, the experimental data suggest that biliary obstruction enhances the inflammatory and microvascular responses of the liver to endotoxemia. Our results provide further support of these findings since the hepatic microcirculatory dysfunction was significantly exaggerated when obstructive jaundice was followed by an LPS challenge. The results also show that the hepatic KCs play a pivotal role in this process.

We used a rodent model to mimic the inflammatory complications of cholestasis at the microcirculatory level. The methods applied in this study enabled us to examine the inter-relationship between various microcirculatory parameters and to compare these events with structural alterations in the liver under different circumstances. First, we found that obstructive jaundice and endotoxemia caused characteristically different microcirculatory responses in the liver. Specifically, LPS induced considerable leukocyte activation, but, this was not accompanied by a severe hepatic perfusion failure. In contrast, BDL resulted in a significantly reduced capillary perfusion, and the leukocyte accumulation was only moderate. A strong causal relationship has been suggested between leukocyte

sticking and decreased capillary perfusion. It is difficult to compare different models and different time frames, but our observations do not support this generalized statement. Nonetheless, the observation time with the presented BDL model is prolonged and it can not be excluded that earlier microvascular perfusion alterations are followed by PMN-induced hepatic inflammation in later phases. Indeed, an increased adhesion molecule expression and PMN adherence were observed only in the later stages of obstructive jaundice. Nonetheless, our data suggest characteristically different microcirculatory responses in the time frame of the present studies.

In our previous study with 24 h of endotoxemia and 6 days of BDL, we demonstrated that histological liver injury is preceded by functional changes. Our present data permit an evaluation of the impact of the different microcirculatory changes on the final outcome of liver injury hallmarked by histological damage. The most severe structural alterations in all liver parts were caused by BDL, which was accompanied primarily by a hepatic perfusion failure, and not by inflammatory reactions (*i.e.* neutrophil activation and accumulation, and TNF-α release). Moreover, the microcirculatory consequences of a 3-day BDL were exacerbated by 1-h endotoxemia (a perfusion deficit, the tissue accumulation of leukocytes and the release of proinflammatory cytokines). Interestingly, these changes were not manifested in considerable alterations at the histological level in the short run. Even so, these observations suggest the impact of impaired tissue perfusion over the inflammatory reactions on the histological injury formation in the examined time frame. As cumulative injury should be presumed with all forms of BDL, the appraisal of these alterations requires careful consideration.

All the models used in these studies were accompanied by a considerably increased KC activation, which could be partially blocked with $GdCl_3$. The blockade elicited by this administration, however, beneficially influenced most of the examined parameters, such as the leukocyte adhesion and migration, the TNF-α release, the HO and MT activities and the structural damage in the periportal region, even in the cases with the most severe combined challenges.

It has been demonstrated that the KCs are primary sources of circulating TNF-α and IL-6 in response to LPS. Additionally, both experimental and clinical observations indicate that the KCs are involved in the increased cytokine secretion in obstructive jaundice. In accordance with these findings, we found excessive proinflammatory cytokine release after LPS administration in BDL animals, accompanied by an increased KC activity. Moreover, our present data suggest that the higher susceptibility to endotoxemia during obstructive jaundice involves KC activation. This suggests a sensitizing triggering mechanism which can lead to the exaggeration of inflammation after repeated stimuli or if the injury is prolonged. Some authors emphasize the direct effect of a biliary obstruction on the hepatocytes and KCs. Others have underlined the importance of the elevated LPS-binding protein in the pathomechanism. This latter hypothesis is supported by the studies of Minter *et al.*,

showing that KCs isolated from experimental animals with BDL are exquisitely sensitive to LPS-binding protein, but not to LPS.

The biochemical parameters also show characteristic changes. The sensitivity of the liver to inflammatory conditions is illustrated by the fact that the MT and HO gene expressions are more inducible in the liver than in other organs. As the production of these proteins is mostly regulated at the transcriptional level, the expressions of their genes and not their protein levels were subjected to examination in our present models. Such regulation of these genes was apparent in these observations: only the induction of the HO-1 and MT-2 isoforms changed considerably, with the induction level dependent on the stimulus applied. As concerns HO-1, its increased expression is regarded as a sign of the activation of an endogenous protective reaction in response to oxidative stress. In our study, a moderately increased induction of hepatic HO-1 was observed even as late as 3 days after the BDL. It is noteworthy, however, that the level of HO-1 induction was higher in the acute phase of endotoxemia than in the later phase of obstructive jaundice. The HO-2 changes demonstrated a similar tendency, with markedly lower induction levels. It is reasonable to assume that endotoxemia alone results in a close to maximal induction of the HO gene, since these changes could not be further enhanced when the BDL and LPS challenges were combined. Furthermore, our measurements were performed at a stage of endotoxemia when the TNF-α levels had attained their maximal values; the elevations in level of this proinflammatory cytokine are effectively prevented by this method of KC blockade. Since TNF-α is a potential enhancer of HO-1 mRNA expression, the lower extent of HO-1 induction may also result from the reducing effect of KC blockade on the TNF-α expression. Hence, the lower induction of HO-1 in the presence of $GdCl_3$ is most probably a consequence of a potentially reduced hepatic injury, as a direct inhibitory effect of $GdCl_3$ on HO-1 gene expression has not been described.

Another manifestation of the stress-induced hepatic response is induction of the MT gene, which has been described in the early phase of endotoxemia and in biliary obstruction. To the best of our knowledge, the isoform-specific changes in hepatic MT expression observed under the above circumstances in the present study are described for the first time here. Similarly to the HO-1 changes, the induction of MT-2 (but not MT-1) peaked after acute endotoxemia and was not enhanced when LPS was combined with BDL. The dependence of the expression of MT-2 on the time course of BDL can not be ruled out (and could not be assessed in the present study); nonetheless, the induction of this gene was not found to be elevated 3 days after BDL. Further studies are needed to elucidate why additive effects of these challenges are not observed for the above gene (HO and MT) expressions, even though LPS has been shown to worsen the consequences of BDL both experimentally and in clinical practice.

In response to GdCl$_3$ treatment, similar isoform-specific elevations in MT-2 induction have been observed, but these were virtually independent of the noxious stimulus. It is conceivable that the mechanisms of MT induction in endotoxemia and after GdCl$_3$ are different. It is likely that the MT mRNA expression elevation is related to the injury caused by free radicals in the case of endotoxemia, whereas MT induction is a direct consequence of the metal ion overload caused by the heavy metal ion Gd^{3+}. The MT proteins are believed to be protective in nature, representing an intrinsic protective mechanism in endotoxemia as well as against heavy metal-induced oxidative damage and against cadmium toxicity. The exact consequences of MT-2 induction are also a potential subject of further examinations, targeting the questions of whether (1) only the MT-2 expression remains elevated, (2) binding between GdCl$_3$ and MTs still exists 24 h after the treatment, and (3) if so, how it influences the free radical-scavenging capacity of MT.

In our experiments, all of the noxious stimuli led to a similar, albeit moderate degree of hepatic DNA single-strand break formation. An increased level of LPO was observed only when BDL and LPS were combined. GdCl$_3$ alone caused elevations in both parameters (particularly in LPO). The hepatotoxic effects of GdCl$_3$ have been reported elsewhere. Our models demonstrated that the free radical-derived hepatotoxicity overwhelms the endogenous hepatic protective antioxidant mechanisms. This is manifested in the characteristic changes in the CAT and Mn-SOD activities, which may reflect the cumulative effect of free radical toxicity, referring also to the time frame of the challenges. With respect to the CAT activity, BDL and BDL combined with LPS comprise a stronger signal than acute endotoxemia alone. Our results support observations that endotoxemia leads to a decrease in hepatic CAT activity and we additionally observed a simultaneous increase in Mn-SOD activity. Most BDL studies have yielded similar results concerning CAT activity, but usually the total SOD activities are determined. In our study with BDL, the mitochondrial Mn-SOD activity was found to increase, with a simultaneous decrease in the cytosolic Cu/Zn-SOD level. We also determined the changes in other components of the superoxide-targeting antioxidant machinery, the levels of reduced glutathione and glutathione peroxidase, but these varied only insignificantly in response to any of the challenges applied. Despite its mentioned toxic effects, a moderate protective effect of GdCl$_3$ was revealed in its partial restoration of the CAT levels in our two models involving endotoxemia.

In view of our present data, the role of GdCl$_3$ is rather controversial. GdCl$_3$ induces some degree of subcellular damage (reflected by the LPO), but despite the hepatotoxic effect attributed to this compound, it has been demonstrated to alleviate the injury caused by agents such as cadmium chloride or retinol. The toxicity of Gd^{3+} depends on the dose and the chemical form (hydroxylated/protein complex form). Our present data suggest that it would also be important to establish whether any effect of GdCl$_3$ on the antioxidant CAT levels is related to its own toxicity or to

its action on the basic challenge models themselves. We believe that the protective effects of GdCl$_3$ (earlier shown to reduce microcirculatory failure, microcirculatory inflammation, structural damage and inflammatory cytokine release) reflect the obvious predominance of the beneficial effects of this compound. The unfavorable subcellular effects, however, warrant caution.

Our present observations should finally be discussed in view of the final end-points of hepatic injury. We believe that the final outcome of the challenges applied corresponds strongly with the functional and structural impairment of the liver. The clinical observations supported the previous demonstration that the histological structural damage and functional (*e.g.* microcirculatory) impairment of the liver caused by biliary obstruction are aggravated by endotoxemia. Acute endotoxemia causes a lesser degree of injury and KC blockade with GdCl$_3$ beneficially influences most of the consequences of the above challenges. In the present study, we also targeted the endogenous antioxidant activities (SOD and CAT), together with an isoform-specific assessment of other proteins which also possess free radical-scavenging properties (HO and MT). The results demonstrate that 2 h of endotoxemia comprises a stronger signal for the induction of a stress response (*e.g.* HO-1 and MT-2) than that of a 3-day biliary obstruction (despite the similar degree of hepatic DNA and membrane damage). The combination of these challenges results in more severe alterations only in LPO. We have also established that, although GdCl$_3$ induces some degree of LPO and DNA damage independently of the various challenges, its alleviating effects are also evident, as the stress-induced induction of HO-1 is reduced and the CAT levels are partially restored in the presence of this intervention. These signs of ameliorated stress-induced hepatic reactions provided by GdCl$_3$ may contribute to its beneficial hepatic protective effects.

6. SUMMARY OF NEW FINDINGS

1. The inflammatory complications of obstructive jaundice are greatly deteriorated if a second hit of endotoxemia is elicited. These inflammatory reactions are manifested in an impairment of the microcirculatory perfusion, the activation and accumulation of leukocytes and enhanced proinflammatory cytokine release.
2. A causal relationship between the hepatic microcirculatory and histological changes after biliary obstruction can be assumed, whereas inflammatory reactions (leukocyte activation) may exert only a relatively minor influence in these factors.
3. Acute endotoxemia comprises a stronger signal for the induction of a stress response (*e.g.* HO-1 and MT-2) than that of a 3-day biliary obstruction (despite the similar degree of hepatic DNA and

membrane damage). The combination of these challenges results in more severe alterations only in LPO.

4. KC blockade with $GdCl_3$ reduces the detrimental microcirculatory consequences of endotoxemia, and beneficially influences the inflammatory reactions when obstructive jaundice is combined with an LPS challenge. The $GdCl_3$ treatment ameliorated the KC and leukocyte activations, the TNF-α release and the tissue accumulation of the PMNs.

5. $GdCl_3$ alone induces some degree of LPO and DNA damage independently of the various challenges, but its alleviating effects are also evident, as the stress-induced induction of HO-1 is reduced and the CAT levels are partially restored in the presence of this intervention.

6. KC blockade ameliorates inflammatory complications of obstructive jaundice by beneficially influencing the microvascular inflammatory reactions with simultaneous positive effects on hepatic biochemical processes and structural injury.

7. ACKNOWLEDGEMENTS

List of full papers related to the subject of the thesis:

I.   **Ábrahám S**, Szabó A, Kaszaki J, Varga R, Eder K, Duda E, Lázár G, Tiszlavicz L, Boros M, Lázár G Jr. Kupffer cell blockade improves the endotoxin-induced microcirculatory inflammatory response in obstructive jaundice. *Shock* 30: 69-74, 2008 (**IF: 3.394)**.

II. **Ábrahám S**, Szabó A, Paszt A, Duda E, Lázár G, Lázár G ifj: A Kupffer-sejt gátlás hatása az endotoxin indukálta gyulladásos válaszreakciókra és a máj mikrokeringési változásaira kísérletes obstrukciós icterusban. *Magyar Sebészet* 62: 298-303, 2009.

III. **Ábrahám S**, Hermesz E, Szabó A, Ferencz A, Duda E, Ábrahám M, Lázár G, Lázár G Jr. The effects of Kupffer cell blockade on the hepatic expression of metallothionein and heme oxygenase genes in endotoxemic rats with obstructive jaundice (submitted for publication).

List of abstracts related to the subject of the thesis:

I.   **Ábrahám S**, Szabó A, Lázár G, Lázár G Jr. Kupffer cell blockade ameliorates the obstructive jaundice-induced microcirculatory inflammatory reaction in the liver. *European Surgical Research* 28: 1-124, 2005.

II. **Ábrahám S**, Ferencz A, Szabó A, Lázár G, Boros M, Hermesz E, Lázár G Jr. Hepatic expression and regulation of metallothionein and heme oxygenase genes in endotoxemic rats with obstructive jaundice – the role of Kupffer cells. *European Surgical Research* 39: 1-90, 2007.

III. Lázár G Jr, **Ábrahám S**, Szabó A, Kaszaki J, Duda E, Lázár G, Tiszlavicz L, Boros M. Kupffer cell blockade improves the endotoxin-induced microcirculatory inflammatory response in obstructive jaundice. *Shock* 29 Suppl 1, 2008.

IV. **Ábrahám S**, Hermesz E, Ferencz A, Szabó A, Lázár G, Boros M, Lázár G Jr. Antioxidant defense system in endotoxemic rats with obstructive jaundice. *Shock* 29 Suppl 1, 2008.

List of full papers not related to the subject of the thesis:

I.   Lázár G, Kaszaki J, **Ábrahám S**, Horváth G, Wolfárd A; Szentpáli K; Paszt A; Balogh Á, Boros M. Thoracic epidural anesthesia improves the gastric microcirculation during experimental gastric tube formation. *Surgery* 134: 799-805, 2003 (**IF: 2.611)**.

II. Lázár G, Kaszaki J, **Ábrahám S**, Wolfárd A, Horváth G, Szentpáli K, Paszt A. A thoracalis epiduralis anaesthesia javítja a gyomor mikrokeringését kísérletes csőgyomor képzés során. *Magyar Sebészet* 56: 25-30, 2003.