Compensatory hepatic regeneration after mild, but not fulminant, intraperitoneal sepsis in rats

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Weiss, Yoram G., Lisa Bellin, Patrick K. Kim, Kenneth M. Andrejko, Charlotte A. Haaxma, Nichelle Raj, E. Elizabeth Furth, and Clifford S. Deutschman. Compensatory hepatic regeneration after mild, but not fulminant, intraperitoneal sepsis in rats. Am J Physiol Gastrointest Liver Physiol 280: G968–G973, 2001.—Sepsis is the leading cause of death in surgical intensive care units. Although both mild sepsis secondary to cecal ligation and single puncture (CLP) and fulminant, double puncture CLP (2CLP) may provoke hepatic death, we hypothesize that regeneration compensates for cell death after CLP but not 2CLP. In male Sprague-Dawley rats, hepatic necrosis, as determined by serum a-glutathione S-transferase (a-GST) levels, was significantly but equally elevated over time after both CLP and 2CLP. Apoptosis, evaluated using both terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and morphological examination, was minimal after both CLP and 2CLP. Regeneration, assayed by staining tissue for incorporation of exogenously administered bromodeoxyuridine, was present after CLP but not after 2CLP. To further substantiate impaired regeneration, steady-state levels of mRNAs encoding JunB, LRF-1, and cyclin D1 were determined. After 2CLP, the absence of JunB, LRF-1, and cyclin D1 mRNAs confirmed failed activation of the mitogen-activated protein kinase-linked proliferative pathway and progression through the cell cycle. Therefore, failed hepatocyte regeneration may be a manifestation of hepatic dysfunction in fulminant sepsis.

SEPSIS AND THE RELATED SYSTEMIC inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) are the leading causes of death in critically ill surgical patients (3, 5). Both involve disordered metabolism (2, 16, 29, 35). Because the liver plays an essential role in the orchestration of complex metabolic responses, hepatic dysfunction is an important component of these syndromes. The liver is the second most commonly affected organ in sepsis/SIRS/MODS, and, as hepatic processes fail, liver cells can be lost (5, 13, 38). The pathophysiological mechanisms leading to hepatocyte dropout in late sepsis remain unknown.

Hepatocyte death can occur via either necrosis or programmed cell death (apoptosis) and has been described in a number of clinically relevant settings such as ischemia-reperfusion, organ transplantation, or toxic injury (17, 22, 26, 27, 39). However, the liver is unique in that it retains the capacity to regenerate in adult life (11, 19, 27, 33). In most settings in which cell loss is accelerated, compensatory mechanisms that include regeneration are activated so that overt hepatic failure is unusual. The role of hepatic regeneration in sepsis, SIRS, and MODS, however, has not been investigated.

In previous studies, we have investigated a murine model of sepsis, cecal ligation and puncture (CLP), with specific reference to hepatic dysfunction. When this model involves a single cecal puncture, resulting in mild sepsis, we have shown that transcription of a number of liver-specific genes is decreased transiently and mortality is essentially nil (2, 2, 14, 15). In addition, we have shown enhanced expression of the interleukin (IL)-6-dependent acute phase reactant a2-macroglobulin and the IL-6-linked transcription factor Stat-3 (1). However, when fulminant, highly fatal sepsis is induced via cecal ligation and double puncture (2CLP), decreased transcription is persistent, and Stat-3 activation/a2-macroglobulin expression abruptly decrease. In addition to its effect on acute phase gene expression, IL-6 also is an essential activator of hepatocyte proliferation/regeneration (11, 50). Recent studies indicate that this process is IL-6 dependent but Stat-3 independent and involves activation of mitogen-activated protein (MAP) kinase-linked transcription of genes such as those encoding JunB and LRF-1 (24). Therefore, in this study, we investigated hepatocyte death and regeneration after CLP and 2CLP. Specifically, we tested the hypothesis that CLP-induced hepatocyte necrosis and apoptosis are compensated for by...
regeneration. In contrast, after 2CLP, we propose that regeneration fails. Furthermore, we hypothesize that the mechanism underlying this difference involves a decrease in the expression of the MAP kinase-linked immediate-early genes junB and lrf-1 and the gene encoding the cell cycle modulator protein cyclin D1.

MATERIALS AND METHODS

Induction of sepsis. All animal studies were approved by the University Laboratory Animals Resources committee of the University of Pennsylvania and conformed to National Institute of Health standards for laboratory animals. Rats were injected subcutaneously with 50 mg/kg of bromodeoxyuridine (BrdU; 2 mg/ml in saline) at the time of the initial operation. Under methoxyflurane anesthesia, reversible sepsis was induced in male, adolescent (250–275 g) Sprague-Dawley rats (Charles River, Boston, MA) via cecal ligation and single 18-gauge puncture (CLP) as described previously (1, 2, 14, 25). Fulminant sepsis was induced with 2CLP (1, 2, 14, 25). Sham-operated (SO) animals served as controls. After the procedure, animals were fluid resuscitated with 50 ml/kg sterile saline injected subcutaneously, awakened, and allowed free access to water and food. At time 0 (unoperated control) and at 3, 6, 16, 24, 48, and 72 h after surgery, rats were reanesthetized with a 40 mg/kg intraperitoneal injection of pentobarbital sodium. Inferior vena cava blood was obtained to determine α-glutathione S-transferase (α-GST) levels, and animals were killed via exanguination. In one set of rats, liver tissue was perfused fixed with 2% paraformaldehyde and harvested for immunohistochemical detection of BrdU incorporation. In a second set of animals, the liver was fixed with 10% formalin for apoptosis staining using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. In a third set, total RNA was isolated as previously described (1, 2, 14, 15, 25).

Determination of α-GST levels in vena caval blood. Blood was collected from the inferior vena cava, placed in a serum separator tube (Becton-Dickenson, Franklin Lakes, NJ), and allowed to coagulate. Samples were spun at 13,000 rpm for 5 min, and the aqueous layer was isolated and stored at −70°C. α-GST levels were determined in duplicate from serum using a quantitative enzyme immunoassay (GST EIA; Biotrin International, Newton, MA; see Refs. 6, 37, and 44). Samples from six animals at each time point were averaged.

Apoptosis detection. In situ cell death was evaluated using TUNEL (Boehringer Mannheim; see Refs. 21, 22, and 32). Paraffin-embedded tissue was fixed in 10% formalin, de-waxed, rehydrated in an ethanol gradient, treated with proteinase K, blocked, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The fluorescein-containing TUNEL reaction mixture, diluted 1:1 with PBS, was incubated on sections for 1 h. TdT was used to enzymatically label the 3′-OH DNA breaks with dUTP. Sheep anti-fluorescein antibody conjugated with horseradish peroxidase was added and further bound to metal-enhanced diaminobenzidine (DAB; Pierce, Rockford, CA) for light microscopic evaluation. The presence of apoptotic hepatocytes was evaluated by an independent pathologist (E. E. Furth), and apoptosis was designated as present only if cells contained both condensed chromatin and positive TUNEL staining.

Detection of cell division/regeneration. Paraffin-embedded, paraformaldehyde-fixed tissue was de-waxed, rehydrated, quenched for endogenous peroxidase activity, and blocked with horse serum/PBS and avidin-biotin blocking solution. Sections were then incubated with a mouse monoclonal primary antibody to BrdU (Sigma, St. Louis, MO) for 45 min, followed by a horse anti-mouse secondary antibody for 30 min (11, 18, 30). Chromogenic staining was performed with DAB as previously described (18).

Northern blot hybridization analysis. Northern blot hybridization analysis was performed on total RNA samples isolated from liver harvested 0, 3, 6, 16, 24, 48, and 72 h after SO, CLP, or 2CLP as previously described (1, 2, 14, 25). cDNAs complementary to the mRNAs encoding the MAP kinase-activated immediate-early genes JunB and LRF-1, the cell cycle protein cyclin D1, and the constitutively expressed β2-macroglobulin were 32P labeled, and blots were hybridized for 16 h. Membranes were washed, and autoradiography and densitometry were performed. Densitometric data for each gene of interest were divided by the density for β2-macroglobulin at the same time point. Studies were performed on RNA obtained from three rats at each time point, and means and SD were calculated.

Data analysis. Cells incorporating BrdU were counted by two independent observers examining six different low-power fields. Apoptosis was evaluated by an independent pathologist. The highest and lowest counts were excluded. Statistical significance (P < 0.05) for cell counts and densitometric data were determined using ANOVA with the Bonferroni test of post hoc significance.

RESULTS

Outcome. As in previous studies (1, 2, 14, 25), SO, CLP, and 2CLP provoked different clinical responses. SO animals recovered uneventfully. CLP was followed by signs of mild sepsis, including lethargy, decreased spontaneous movement, and poor grooming. All resolved within 48 h. Six to sixteen hours after 2CLP, rats exhibited decreases in food intake, spontaneous movement, and grooming. Diarrhea, piloerection, tachypnea, and extreme lethargy were evident by 16 h.

Outcome data paralleled previous studies (1). Six unoperated controls, the 36 SO animals, and the 36 CLP animals all survived. In contrast, mortality was high after 2CLP. Between 16 and 24 h, mortality was 50%, between 24 and 48 h 75%, and between 48 to 72 h 90%.

Hepatic necrosis after CLP and 2CLP. Figure 1 depicts α-GST serum levels after SO, CLP, and 2CLP. Relative to SO and time 0, α-GST levels were significantly elevated 16, 24, 48, and 72 h after both CLP and
Fig. 2. Apoptosis 3, 6, 16, 24, 48, and 72 h after CLP, 2CLP, and SO. Apoptotic nuclei identified by morphology and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Representative apoptotic nuclei indicated by arrows. CV, central vein.

Fig. 3. Regeneration 24, 48, and 72 h after CLP, 2CLP, and SO. Bromodeoxyuridine (BrdU) was administered subcutaneously at the time of the initial operation. Detection was performed by using mouse monoclonal primary antibody to BrdU, a horse anti-mouse secondary antibody, and chromogenic staining with diaminobenzidine.
2CLP. \(\alpha\)-GST levels were significantly higher in the 2CLP group than in CLP animals at 24, 48, and 72 h.

**Hepatic apoptosis induced by CLP and 2CLP.** A small number of TUNEL and morphologically positive apoptotic cells was detected between 6 and 48 h after both CLP and 2CLP (Fig. 2). Apoptosis was evident in both hepatocytes and nonparenchymal cells. There was no difference in the number of apoptotic cells per high-powered field between CLP and 2CLP. A few apoptotic cells were detected after SO.

**Hepatic regeneration after CLP and 2CLP.** BrdU uptake was evident 24 h after single-puncture CLP. At this time point, 20 ± 4 cells/low-powered field were BrdU positive. This increased to 28 ± 6 cells/low-powered field and 34 ± 7 cells/low-powered field at 48 and 72 h after CLP, respectively (Fig. 3). Fewer than five BrdU-positive cells per low-powered field were evident at any time point in 2CLP animals.

To investigate the mechanisms underlying depressed regeneration after 2CLP, expression of JunB and LRF-1, MAP kinase-linked immediate-early response genes (24), and cyclin D1, which modulates extracellular mitogenic stimuli to allow progression past the G1 restriction point (8, 40), were examined.

Northern blots are reproduced in Fig. 4A. These data, quantified in Fig. 4B, indicated low levels of expression at time 0 in all experiments and after SO. Hybridization was increased by fivefold 24 h after both CLP and 2CLP. However, although the signals remained intense 48 h after CLP, they reverted to basal levels 48 h after 2CLP. This observed decrease is consistent with the lack of BrdU uptake after 2CLP (Fig. 3).

**DISCUSSION**

In the present study, hepatocyte loss in both CLP and 2CLP occurs via necrosis, reflected in elevated \(\alpha\)-GST levels. Low levels of apoptosis were detected also. Most interestingly, hepatic regeneration, as measured by BrdU uptake, was present after CLP but not after 2CLP.

In previously published data from our laboratory, we have demonstrated the divergence of outcome between CLP and 2CLP (1, 2, 14, 25). Furthermore, we found that CLP induced a transient but profound downregulation of several liver-specific genes (2). This decrease was persistent after 2CLP (14, 25). Pathological changes in the liver were more profound after 2CLP (1,
2, 14, 25). In the present study, hepatic necrosis appears to correlate with this divergence in outcome and transcription. Low levels of α-GST release, however, make it unlikely that this correlation has pathological significance. In contrast, regeneration inversely parallels mortality and the previously described persistent alterations in transcription (1, 14, 25). Additionally, hepatocyte regeneration was evident at later time points than necrosis and apoptosis, suggesting that it is a compensatory process. The decreased expression of JunB, LRF-1, and cyclin D1 is also consistent with our finding of an inverse correlation between cellular regeneration and a selective but irreversible loss of transcription 24 h after 2CLP (1, 14, 25).

Hepatocyte necrosis was evaluated by α-GST serum levels. α-GST is unique to hepatocytes, is rapidly released in the circulation, and therefore is a more sensitive indicator of hepatocellular injury than standard transaminase levels (6, 36, 37, 44, 47).

Although necrosis is a well-known component of late sepsis-associated liver failure, the presence of apoptosis has not been documented universally. Apoptosis is well described after direct hepatotoxic injury, such as that associated with carbon tetrachloride poisoning or after ischemia-reperfusion (10, 41). CLP-induced parenchymal cell apoptosis has been previously described in the lung, gut, muscle, kidney, and lymphoid tissue (20, 21). However, most studies show no evidence of apoptosis in the liver, brain, and heart. Indeed, others report that a second insult, such as galactosamine administration or ischemia, is required for apoptosis to be clearly evident in the liver after inflammatory insults (22, 23, 31, 42, 43, 45). Our studies confirm this, because only low levels of apoptosis could be appreciated. Apoptosis seen in CLP and 2CLP rats indicates that sepsis/MODS may be associated with some up-regulation of programmed cell death, but it is likely that this is a less important process than necrosis.

Cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, have been implicated in the modulation of intrahepatic sepsis-associated responses (1, 4, 9, 13, 28, 34, 49). These cytokines may play a role in cell death and regeneration as well. It is known, for example, that TNF-α and IL-1β have been implicated in inducing cell death via both apoptosis and necrosis (28). However, both exert anti-apoptotic effects via activation of the transcription factor nuclear factor-κB (7, 28, 46, 48). Given the pleiotropic nature of TNF-α/IL-1 mediated responses, it is not surprising that there was only a minor difference in necrosis and apoptosis after CLP and 2CLP. TNF-α and IL-6 are also important components in the early signaling pathways leading to regeneration (33). Cressman et al. (12) found impaired regeneration and increased mortality after 75% hepatectomy in IL-6-deficient mice. Similarly, Yamada et al. (50) documented failed regeneration after hepatectomy in mice deficient in the 55-kDa TNF-α receptor. In both studies, regeneration was restored after a single dose of IL-6. In a previous study, we demonstrated that CLP and 2CLP evoked different IL-6-linked responses (1). Specifically, activation of the IL-6-linked transcription factor Stat-3 and expression of the IL-6-activated acute-phase reactant α2-macroglobulin decreased to nearly undetectable levels between 16 and 24 h after 2CLP but not after CLP. This is clearly consistent with, and might well explain, failed regeneration in the liver after 2CLP but not CLP.

In conclusion, the present study indicates that failed hepatocyte regeneration may be an additional manifestation of hepatic dysfunction in fulminant sepsis. These findings suggest that further studies will clarify the role of liver regeneration in sepsis and may identify an important therapeutic approach.

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