Cell proliferation, apoptosis, NF-κB expression, enzyme, protein, and weight changes in livers of burned rats

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Received 5 May 1999; accepted in final form 14 December 2000

Am J Physiol Gastrointest Liver Physiol 280: G1314–G1320, 2001. —Thermal injury has been shown to alter gut epithelium and heart myocyte homeostasis by inducing programmed cell death. The effect of thermal injury on hepatocyte apoptosis and proliferation, however, has not been established. The purpose of this study was to determine whether a large thermal injury increases liver cell apoptosis and proliferation and whether these changes were associated with alterations in hepatic nuclear factor κB (NF-κB) expression and changes in liver enzymes and amount of protein. Sprague-Dawley rats received a 40% total body surface area scald burn or sham burn. Rats were killed and livers were harvested at 1, 2, 5, and 7 days after burn. Liver cell apoptosis was determined by terminal deoxyuridine nick end labeling (TUNEL) assay and cell proliferation by immunohistochemistry for proliferating cell nuclear antigen. Hepatic NF-κB expression was determined by Western blot, and total hepatic protein content was determined by protein assay. Protein concentration decreased after burn compared with sham controls (P < 0.05). Liver cell apoptosis, proliferation, and NF-κB expression in hepatocytes increased in burned rats compared with controls (P < 0.05). It was concluded that thermal injury induces hepatic cell apoptosis and proliferation associated with an increase in hepatic NF-κB expression and a decrease in hepatic protein concentration.

PRESERVING ORGAN HOMEOSTASIS depends on a balance between cell proliferation and cell death (17). Alterations in the balance between apoptosis and proliferation often cause a change in organ function, integrity, and homeostasis (18). There is evidence that thermal injury induces programmed cell death (13, 20) with a concomitant loss in cellular mass and absorptive surface area (13, 20). Lightfoot et al. (13) demonstrated that thermal injury induces apoptosis in myocardial cells, which impaired cardiac function.

The liver has been shown to play a pivotal role in a thermal injury by modulating immune function, inflammatory processes, and the acute phase response (5, 7, 12). Recent studies suggest that thermal injury affects liver morphology in rats by decreasing protein and DNA concentrations (12). Previously, it was shown that nuclear factor κB (NF-κB) was crucial in liver regeneration (3, 10). NF-κB is a ubiquitous, rapidly acting transcription factor involved in immune and inflammatory reactions (10, 16, 21). It exerts its immune and inflammatory response by regulating expression of cytokines, chemokines, cell adhesion molecules, and growth factors (10, 16, 21). NF-κB in the cytoplasm consists of an inactive DNA binding heterodimer containing a p50 and a protein subunit, either RelA, RelB, or c-Rel. This complex is bound to a cytoplasmic inhibitor (IκB) (10, 16). After stimulation by cytokines, virus infection, or lipopolysaccharides, IκB is degraded to release the NF-κB dimer. Activated NF-κB translocates into the nucleus and binds to DNA to become a regulator of the transcription of NF-κB responsive genes, e.g., cytokines, adhesion molecules, and acute phase proteins (2). The function of NF-κB during liver regeneration is not entirely well defined. However, increased activation of NF-κB has been suggested to promote cell survival and exert anti-apoptotic effects (4, 6, 10). This hypothesis is further supported by Beg et al. (3) in which NF-κB/RelA-deficient knock-out mice experienced fetal death from massive hepatocyte apoptosis.

We propose that liver homeostasis is a primary component of survival and improved clinical outcome after thermal injury. The purpose of this study was to determine whether thermal injury induces liver cell apoptosis or proliferation and whether these changes are associated with alterations in liver enzymes and morphology and hepatic NF-κB activation.

MATERIAL AND METHODS

Eighty adult male Sprague-Dawley rats (Harlan Sprague Dawley, Houston, TX) weighing 300–350 g were housed in wire bottom cages in a temperature-controlled room with a 12:12-h light-dark cycle. All animals were acclimated to their environment for 7 days. Rats received a liquid diet of Sustacal (Mead Johnson Nutritional, Evansville, IN) and water.
ad libitum for the entire study period. All animals were pair-fed. Sixty rats received a 40% total body surface area (TBSA) third-degree burn (8) and were immediately, under resuscitated with intraperitoneal lactated Ringer (60 ml/kg). Twenty animals were anesthetized but received no thermal injury (sham burn) to serve as controls. Four burned rats died within 24 h.

Rats were killed by decapitation at 1, 2, 5, or 7 days after burn. Serum was collected in serum separator tubes and plasma in EDTA tubes, spun at 1,000 g for 15 min, decanted, and frozen at −70°C until analysis. The entire liver was removed, weighed, and sectioned. One section (500 mg) was immediately fixed in 10% buffered formalin, then embedded in paraffin within 48 h.

Hepatic Synthesis

Serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALKP) were measured using a nephelometer (Behring, Deerfield, IL).

Hepatic NF-κB Activation

Cytosolic and nuclear hepatic NF-κB expression were determined by Western blot at days 1 and 2 after burn. Liver tissue (260–300 mg) was homogenized and used for preparation of cytosolic and nuclear extracts. The extracts were stored at −70°C for further analysis. Extracts were thawed and diluted 1:10 for protein concentration determination using a bicinchoninic acid protein assay (BCA; Pierce Chemical, Rockford, IL). Western blot analysis was performed using 50 μg of protein from the cytosolic and nuclear cellular extracts on an 8% acrylamide gel. The gel was electroblotted to a nitrocellulose membrane, incubated with polyclonal rabbit IgG anti-human p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (Bio-Rad Laboratories, Hercules, CA). Bands were detected by enhanced chemiluminescence using ECL Western blot reagents and exposure to ECL hyper-film (Amersham Life Science Products, Arlington Heights, IL). The relative amount of cytosolic and nuclear NF-κB protein was determined by densitometry analysis at 260 nm using a scanning transmission densitometer (Applied Imaging, Santa Clara, CA). Equal loading of the gel was confirmed by stripping and reprobing the membrane for β-actin.

Hepatic Changes

Liver weight and liver weight-to-body weight ratios were obtained. Liver protein concentration was determined by total protein assay (Bio-Rad) based on the method of Bradford.

Immunohistochemistry

Formalin-fixed tissues were processed and embedded into paraffin. Sections 4-μm thick, obtained at 40- to 50-μm intervals, were deparaffinized in xylene and alcohol and then rehydrated in deionized water. Immunohistochemical staining procedures were then performed.

Proliferation: proliferating cell nuclear antigen. Hepatocyte proliferation was determined by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Deparaffinized histological sections were pretreated with proteases and HCl to reduce background staining. Nonspecific binding was diminished by incubating sections with goat serum. Sections were incubated with PCNA-HRP conjugate (Santa Cruz) at a 1:50 dilution overnight at 4°C, 3,3'-Diaminobenzidine (3,3'-DAB) peroxidase substrate was used for color detection. The sections were incubated for 3–6 min under microscopic control followed by counterstaining with Mayer’s hematoxylin.

PCNA-positive cells were counted on two sections from each animal. Two blinded observers examined five high-power fields in four different areas on each section for PCNA-positive cells. Proliferating cells were identified from the red-brown stained nucleus or cytoplasm. All hepatocytes within the field were counted, and proliferation was expressed as the number of proliferating cells per 100 hepatocytes. Values per animal for all high-power fields were averaged to calculate hepatocyte proliferation rate.

Apoptosis: terminal deoxyuridine nick end labeling. The terminal deoxyuridine nick end labeling (TUNEL) method (Apoptag; Oncogene, Baltimore, MD) was used for histologic identification of apoptotic cells in the liver. Protein was digested using protease K (20 μg/ml in PBS) to decrease background contamination. The sections were incubated with reaction buffer (containing digoxigenin-labeled dUTP) and freshly prepared terminal-deoxynucleotidyl transferase solution at 37°C for 2 h. Sections were incubated with anti-digoxigenin peroxidase at room temperature for 30 min followed by a thorough washing with PBS. Application of 3,3'-DAB peroxidase substrate for 3–6 min under microscopic control and counterstaining with Mayer’s hematoxylin was made for color detection.

Two blinded observers counted five high-power fields in four different areas on each section (~5,500 cells) for TUNEL-positive cells. Apoptotic cells were identified as those with a red-brown staining of the nucleus or as apoptotic bodies, which appear as fragments of apoptotic cells engulfed by neighboring epithelial cells.

All hepatocytes within the field were counted, and apoptosis was expressed as the number of apoptotic cells per 1,000

Table 1. Total liver weight, weight per 100 g body weight, and liver protein content

<table>
<thead>
<tr>
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<th>Control (days postburn)</th>
<th>40% TBSA Burn (days postburn)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>13.0 ± 0.9</td>
<td>12.4 ± 0.8</td>
</tr>
<tr>
<td>Liver/body wt, %</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Liver protein content, mg/ml</td>
<td>0.98 ± 0.01</td>
<td>0.97 ± 0.01</td>
</tr>
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Values are means ± SE; n = no. of experiments. TBSA, total body surface area. *Significant difference vs. control at corresponding day, P < 0.05.
hepatocytes. Values for all sections were averaged to calculate hepatocyte apoptosis for each animal.

Apoptosis: active caspase-3. To corroborate the data obtained by TUNEL, staining additional immunohistochemistry with a polyclonal antibody for active caspase-3 (R & D Systems, Minneapolis, MN) was made. The deparaffinized histological sections were treated with proteinase K (30 μg/ml) in Tris-buffered saline (0.05 M TBS, pH 7.8) followed by 0.6% H2O2 in methanol for 10 min to reduce background staining. Antigen retrieval was performed by incubating the slides in Antigen Retrieval Citra Plus (Biogenex, San Ramon, CA) at 90°C for 10 min. Using an automated immunohistochemistry system (Ventana ES; Ventana, Tucson, AZ), we incubated the sections with the polyclonal rabbit caspase-3 active antibody at 1:200 dilution for 32 min at 39°C. Signal detection used a biotinylated secondary goat anti rabbit antibody and an Alkaline Phosphatase Blue kit (Ventana). Slides were counterstained with filtered nuclear fast red (Vector, Burlingame, CA) for 10 min.

Caspase-3-positive cells were counted on two sections from each animal. Five high-power fields in different areas on each section were examined for caspase-3-positive cells. Positive cells were identified by blue staining of the cytoplasm. Values for all high-power fields per animal were averaged, and the results were expressed as the number of caspase-3-positive cells per high-power field.

Ethics and Statistics

The study was approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX) and followed the National Research Council’s guide. Sta-
Statistical comparisons were made by ANOVA and Student’s t-test, with the Bonferroni correction. Data are expressed as means ± SE. Significance was accepted at $P < 0.05$.

RESULTS

Mortality

The mortality rate during the study period, which started 24 h after burn, was 0% with no differences in food intake.

Hepatic Changes

Liver weight increased in thermally injured rats 2 days after burn, whereas liver weight was constant in sham control animals ($P < 0.05$, Table 1). Liver-to-body weight ratio was also increased on day 2 after burn ($P < 0.05$, Table 1). Hepatic protein concentration was significantly decreased during the study period in burned animals compared with controls ($P < 0.05$, Table 1).

Hepatic Enzymes, Protein, and NF-κB Activation

Serum AST and ALT were significantly increased 24 h after burn ($P < 0.05$). These enzymes decreased 2 days after burn and reached control values 5 days postburn. Serum ALKP peaked 2 days after burn and reached control levels at 5 days after injury (Fig. 1, A–C). Cytosolic and nuclear NF-κB/RelA expression (p65) was increased in animals at day 1 and day 2 postburn compared with controls (Fig. 2, A and B). Interestingly, the ratio of nuclear to cytoplasmic concentration did not change, indicating the total cellular amount of NF-κB increased with no detectable increase in activated nuclear NF-κB.

Histology

Representative hematoxylin and eosin-stained liver sections for postburn day 7 indicated absence of necrosis in control and burned rats as shown in Fig. 3, A and B.

Proliferation

The physiological proliferation of hepatocytes is ~0.4–0.8 per 100 hepatocytes. Rats receiving a thermal injury had a higher proliferation rate throughout the study period with its peak at 2 days after burn compared with controls (Fig. 4, A and B, and Fig. 5).

Fig. 3. Representative liver sections (hematoxylin and eosin) of control rat (A) and of burned rat (B) at day 7 after thermal injury. Magnification, $\times 70$.

Fig. 4. A: representative section of the liver of a control rat 2 days after the sham burn. Proliferating hepatocytes were identified as PCNA positive (dark staining). Magnification, $\times 100$. B: representative section of the liver of a rat 2 days after burn. Proliferating hepatocytes were identified as PCNA positive (dark staining). PCNA, proliferating cell nuclear antigen. Magnification, $\times 100$. 
Apoptosis. No inflammatory infiltrate was identified upon histologic examination of the liver. The physiological rate of hepatic apoptosis is 0.3 hepatocytes per 1,000 hepatocytes. The apoptotic index by TUNEL increased as early as 1 day after burn. Hepatocyte apoptosis remained increased 7 days postburn compared with control (*P < 0.05; Figs. 6, A and B, and 7). The levels of active caspase-3, as depicted in Fig. 8, showed an early increase on day 1 (n = 5) and day 2 (n = 5).

DISCUSSION

Cell death occurs by two distinctly different modes: programmed cell death (apoptosis) or necrosis (17, 18). Apoptosis is characterized by cell shrinkage, DNA fragmentation, membrane blebbing, and phagocytosis of the apoptotic cell fragments by neighboring cells or extrusion into the lumen of the bowel without inflammation. This is in contrast to necrosis, which involves cellular swelling, random DNA fragmentation, lysosomal activation, membrane breakdown, and extrusion of cellular contents into the interstitium. Membrane

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**Fig. 5.** Percent of proliferating cells measured by PCNA. *Significant difference between burn vs. control, *P* < 0.05. Data are means ± SE. For burned rats *n* = 7, and for controls *n* = 2, per time point.

**Fig. 6.** A: representative liver section of control rats, 24 h after sham burn. Few apoptotic cells were identified as TUNEL positive (dark staining, arrow). Magnification, ×100. B: representative section of the liver in a rat 24 h after burn. Several apoptotic cells were identified as TUNEL positive (dark staining, arrows). TUNEL, terminal deoxyuridine nick end labeling. Magnification, ×100.

**Fig. 7.** Apoptotic cells measured by TUNEL assay 1, 2, 5, and 7 days after burn expressed as positive apoptotic hepatocytes per one thousand hepatocytes. Burned rats had significantly higher rates of hepatocyte apoptosis compared with controls. Data are means ± SE. For burned rats *n* = 7, and for and controls *n* = 2, per time point. *Significant difference compared with controls at *P* < 0.05.

**Fig. 8.** Apoptotic cells assayed for caspase-3 activation by immunohistochemistry 1, 2, and 7 days after burn expressed as caspase-3-positive staining hepatocytes per high-power field (HPF). Data are means ± SE. *Significant difference compared with controls at *P* < 0.05.
breakdown and cellular content release induce inflammation with the migration of inflammatory cells and release of pro-inflammatory cytokines and free radicals, which leads to further tissue breakdown. There are two established methods by which to identify apoptosis, both of which have been criticized (14). The TUNEL method, which is used in the present study, labels the ends of DNA fragments. However, DNA fragmentation, but to a lesser extent, also occurs in cell necrosis. Thus the TUNEL assay may lightly stain necrotic cells, thus making differentiation between apoptosis and necrosis more difficult. Since necrosis is associated with histologic signs of inflammation, whereas apoptosis is not, the two processes may be differentiated by morphological criteria. We found no evidence of necrosis in the liver in the present study. We, therefore, conclude that the detected DNA fragmentation is most likely due to apoptosis. These findings by TUNEL assay were confirmed by the noted early increase in caspase-3 activation. The decrease in the number of caspase-3-positive hepatocytes on day 7, however, is not contradictory as the activation of caspase-3 is an early event in the apoptotic process.

In the present study we show that thermal injury induces hepatocyte apoptosis. This increase in programmed cell death is accompanied by an increase in proliferation, suggesting a trend toward tissue homeostasis. Despite the compensation for increased apoptosis, increased hepatocyte proliferation does not restore hepatic mass and protein concentration. This is shown as a significant decrease in hepatic protein concentration in burned rats throughout the entire study period. It has been previously shown that burn induces small bowel epithelial cell apoptosis (13) and that small bowel epithelial cell proliferation was not increased, leading to a loss of mucosal cells and hence mucosal mass (20). Similar findings were demonstrated in heart muscle where burn-induced cardiomyocyte apoptosis with an unchanged proliferation rate caused cardiac impairment and dysfunction (13).

The mechanisms by which burn injury induces programmed cell death in hepatocytes has not been defined. Some studies suggest that hypoperfusion and ischemia-reperfusion promote apoptosis (1, 9, 11, 15, 18, 19). In addition, pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) have been described as apoptotic signals (2, 4). We have previously shown that serum and hepatic concentration of pro-inflammatory cytokines such as IL-1α/β, IL-6, and TNF-α are increased after thermal injury (12). Two possible mechanisms for increased hepatocyte apoptosis are decreased splanchic blood flow and elevation of pro-inflammatory cytokines.

The liver has been shown to play a pivotal role after thermal injury by modulating immune function, inflammatory processes and the acute phase response (5, 7, 12). The systemic stress response is an orchestrated attempt to restore homeostasis after trauma (5, 7, 12). Production of acute phase proteins and constitutive hepatic proteins may release survival signals to counteract increased hepatocyte apoptosis by inhibiting apoptosis or stimulating proliferation. NF-κB is involved in the regulation of cell proliferation and shows anti-apoptotic properties (4, 6, 10). It has been suggested that NF-κB plays an important role for liver regeneration (3). In the present study, hepatic cytoplasmic NF-κB expression was increased in thermally injured rats compared with controls. However, nuclear translocation or activation of NF-κB was not increased. Thus the anti-apoptotic effects of NF-κB might not be sufficient to counteract the potential pro-apoptotic stimuli (decreased perfusion, cytokines).

Even with indications of increased hepatocyte apoptosis and proliferation, there is further strong evidence that thermal injury causes liver damage. In rats, serum AST, ALT, and ALKP significantly increased after burn when compared with controls. It was observed that serum AST and ALT peaked during the first day postburn and ALKP during the second day postburn; however, all enzymes returned to baseline between 5 and 7 days postburn. This liver damage may be associated with increased hepatic edema formation. In the present study the liver weight and liver-to-body weight ratio significantly increased 2–7 days after burn compared with controls. As total hepatic protein concentration was significantly decreased in burned rats, we suggest that the liver weight gain may be due to increased edema formation rather than increases in the number of hepatocytes or protein levels.

In conclusion, burn injury induces programmed cell death in hepatocytes with a concomitant increase of hepatocyte proliferation. Despite an upregulation of hepatocyte proliferation, hepatic protein content was decreased after a thermal injury. These changes are associated with an increase in hepatic cytoplasmic and nuclear NF-κB expression.

We thank Shanaz Quadeer and Ann S. Burke for valuable technical support.

This study was supported by Shriners North America Grant 8010.

REFERENCES


