

Mechanism of Cell Death After Extensive Liver Resection: Apoptosis or Necrosis?

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Citation

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Abstract

Introduction: Liver resection of more than 80% induce hepatic failure but the mechanism of the remnant liver dysfunction has not been clarified in detail yet. Apoptosis and necrosis are two different cell death mechanisms. Apoptosis is a fundamental biologic process that is crucial in several physiologic and pathophysiologic processes of the liver. In this study, we analysed mechanism of the liver injury after lethal/extensive hepatectomy in rats.

Material and method: Forty Wistar male rats weighing 200 to 250 gm divided into three groups: control group (sham laparotomy, n=10), 70% hepatectomy group (n=15), and 85% hepatectomy group (n=15). All rats underwent relaparotomy 24h later. Liver injury, hepatocyte necrosis and apoptosis were assessed.

Results: Liver damage and hepatocyte apoptosis were increased significantly in the 85% hepatectomy group

Conclusion: Liver failure after extensive hepatectomy was characterized by increased apoptosis.

INTRODUCTION

Hepatic failure after liver resection in rats increases markedly beyond the classic 2 / 3 resection. Hepatic failure occurs as a result of the remnant liver dysfunction^{1,2}.

Liver resection of more than 80% induce hepatic failure, but the mechanism of the remnant liver dysfunction after extensive hepatectomy has not been clarified in detail³.

Apoptosis and necrosis are two different cell death mechanisms. Apoptosis, often synonymously used with the term “programmed cell death”, is an active, genetically controlled process that removes unwanted or damaged cells.

Apoptosis is a fundamental biologic process that is important in many physiologic and pathophysiologic processes in the liver^{4,5}.

For a long time necrosis was considered to be an alternative mechanism to apoptosis. However, recent data indicate that, in contrast to necrosis caused by very extreme conditions. There are many examples when this form of cell death may be a normal physiological and regulated event. Furthermore, signaling pathways, kinase cascades, and mitochondria,

participate in both processes and it is possible of these processes to switch to an other⁶.

The aim of this study was to assess mechanism of the liver injury after lethal and nonlethal hepatectomy in rats.

MATERIAL AND METHOD

ANIMALS

Fourty Wistar male rats weighing 200 to 250 gm at the beginning of the experiments were used. This experiment was reviewed by the Committee on Ethics in Animal Experiments of the Ankara University Faculty of Medicine. Before each operation, animals were fasted overnight with free access to water.

EXPERIMENTAL STUDY

The rats were divided into three groups: control group (sham laparotomy, n=10), 70% hepatectomy group (n=15), and 85% hepatectomy group (n=15). Anesthesia was induced by subcutaneous injection of a mixture of ketamine hydrochloride 100 mg/kg and xylazine 25 mg/kg. The abdomen was opened, the liver was mobilized and gently manipulated, and abdomen was closed in control group.

Seventy percent hepatectomy included removal of left lateral and median lobes. Eighty-five percent hepatectomy included removal of left lateral, median, caudate anterior, and part of right superior lobes (according to Anderson and Higgins).

All rats underwent relaparotomy 24h later. After fixation in formalin buffered solution, remnant liver tissues harvested for histopathologic and immunohistochemical examination. All the blood samples for biochemical analyses were drawn with cardiac puncture.

All rats underwent relaparotomy 24h later. Remnant liver tissues were harvested for histopathologic analysis and TUNEL assay.

HISTOPATHOLOGY

After washing out the blood from the right lobe of the liver by infusion of normal saline via portal vein cannula, 0.2 mM trypan blue (Sigma Chemical Co., St. Louis, MO, USA) was infused for 10 minutes through the same cannula. Excess dye was removed by perfusion of normal saline for an additional 5 min. Then livers were perfused with 1% paraformaldehyde for 6 min., and fixed tissue was embedded in paraffin and processed for light microscopy. Six μ m sections were deparaffinized and counterstained with eosin to identify dead cells marked with trypan blue.

TUNEL ASSAY

For this assay, the manufacturer's instructions (ApopTag Peroxidase In situ Apoptosis Detection kit, Intergen Co., NY) were followed. Briefly, tissue sections deparaffinized, the tissue sections in six micron thickness in a coplin jar by 3 changes of xylene and 2 changes of absolute ethanol for 5 minutes of each change, the slides were washed once in 95 % ethanol and once in 70% ethanol for 3 minutes of each wash. After this, all slides were washed in PBS for 5 minutes.

The pretreatment of the slides was made by protein digesting enzyme for 15 minutes at room temperature. After pretreatment, the slides were washed in 2 changes with the distilled water for 2 minutes, each wash.

Endogenous blockage was made by 3% hydrogen peroxide in PBS for 5 minutes at room temperature. After, the slides were washed twice with PBS for 5 minutes of each time.

Equilibration buffer were applied on each slide for 20 seconds. After the tapping off excess liquid, All slides were incubated at 37°C with TdT enzyme for 1 hour. The reaction was stopped by stop/wash buffer and the slides were washed

in 3 changes of PBS for 1 minute in each wash. After the tapping off excess liquid, the slides were incubated with anti-digoxigenine peroxidase conjugate at room temperature for 30 minutes. Then the slides were washed with 4 changes of PBS for 2 minutes in each wash at room temperature. The staining was made by DAB for 5 minutes. After the staining and washing the slides, The counterstaining was made by methyl green for 10 minutes. Then, the slides were washed in 3 changes of distilled water in each wash. Finally, All slides were washed with 100% N-butanol in coplin jar.

EVALUATION OF CELL NECROSIS

The number of necrotic hepatocytes were counted in 20 high power (x400) fields using videomicroscope. 1000 hepatocytes were counted in asinary zone I and determined the ratio of positive painted nucleus. All histological evaluations were done in a blinded fashion.

EVALUATION OF APOPTOSIS

The number of apoptotic hepatocytes were counted in 20 high power (x400) fields using videomicroscope. 1000 hepatocytes were counted determined the ratio of positive painted cells. All histological evaluations were done in a blinded fashion.

SERUM LEVELS OF LIVER ENZYMES

All the blood samples for biochemical analyses were drawn with cardiac puncture.

The degree of hepatic injury was assessed by serum levels of serum alanine aminotransferase (ALT) , aspartate aminotransferase (AST) , Alkaline phosphatase (ALP) total and direct bilirubin.

All the blood analyses were performed by Hitachi 747(Boehringer Mannheim)

HISTOLOGICAL ASSESSMENT OF HEPATOCELLULAR INJURY

The degree of hepatic injury was graded from 0 to 3 that described by Yamakawa et al.,

- Grade 0 : no morphologic evidence of injury.
- Grade 1 : scattered pericentral necrosis seen uniformly, but only within a five-hepatocyte circumference around the central vein.
- Grade 2 : pericentral necrosis seen uniformly, but only within a five-hepatocyte circumference around the central vein, with the other areas

undamaged.

- Grade 3 : widespread hepatocellular necrosis, extending beyond a five hepatocyte circumference around the central vein, frequently reaching to the midzone.

STATISTICAL ANALYSIS

Data were expressed as mean \pm SD unless otherwise stated. All the statistical analyses were made using a commercial statistical software package (SPSS for Windows release 6.0. SPSS Inc. Chicago, Ill). Comparison among multiple groups were performed with one-way ANOVA followed by Bonferroni post-hoc tests. A probability value less than 0.05 was considered to be statistically significant.

RESULTS

SERUM LEVELS OF LIVER ENZYMES

Chemistry profiles for ALT;AST;ALP and total/direct bilirubin are presented in Table 1. Serum levels of ALT;AST;ALP and total/direct bilirubin in the 85% hepatectomy group were higher than 70% and control groups ($p < 0.05$). Serum total and direct bilirubin levels of the 70% hepatectomy group were higher than control group but difference was not significant. ALT, AST and ALP levels in 70% hepatectomy group were higher than control group and difference was significant ($p < 0.05$).

Figure 1

Table 1: Serum chemistry profiles for ALT;AST;ALP and total/direct bilirubin

Groups	Control Mean \pm SD Median	% 70 Hepatectomy Mean \pm SD Median	%85 Hepatectomy Mean \pm SD Median
Total Bilirubin (mg/dl)	0.5 \pm 0.1 0.4	0.7 \pm 0.4 0.5	2.1 \pm 0.4 0.5
Direct Bilirubin (mg/dl)	0.3 \pm 0.1 0.2	0.3 \pm 0.1 0.2	0.9 \pm 0.2 0.9
Indirect Bilirubin (mg/dl)	0.2 \pm 0.1 0.1	0.3 \pm 0.3 0.2	1.2 \pm 1 0.9
AST (IU/L)	166 \pm 40 157	1245 \pm 500 1248	3451 \pm 1320 3505
ALT (IU/L)	58 \pm 5 58	770 \pm 354 771	2142 \pm 1044 2390
ALP (IU/L)	239 \pm 62 219	608 \pm 226 559	725 \pm 226 632
Apoptotic Cell Count	17.3 \pm 9 14	27.3 \pm 12 19	57 \pm 15 20
Necrotic Cell Count	5.2 \pm 4 4	42 \pm 25 15	44 \pm 23 33

ALT:alanine aminotransferase;AST aspartate aminotransferase;ALP: Alkaline phosphatase

APOPTOTIC AND NECROTIC CELL COUNT

Necrotic cell count in 70% and 85% hepatectomy groups was higher than control group ($p < 0.05$) but difference between 70% and 85% groups was not significant ($p > 0.05$). Apoptotic cell count in 70% and 85% hepatectomy groups was higher than control group ($p < 0.05$) and apoptotic cell count in 85% hepatectomy was higher than 70% hepatectomy group and difference was significant ($p < 0.05$).

LIVER INJURY GRADE

Liver injury grade of groups presented in Table 2. Grade 2 injury most frequently seen in 70% hepatectomy group and grade 3 injury most frequently seen in 85% hepatectomy group.

Figure 2

Table 2: Liver injury grades

	GRADE 0 (%)	GRADE I (%)	GRADE II (%)	GRADE III (%)
Control	60	30	10	0
70% Hepatectomy	0	47	53	0
85% Hepatectomy	0	66	7	27

Figure 3

Figure 1: Apoptotic hepatocytes in 70% hepatectomy group (x460). (Arrows indicates apoptotic hepatocytes.)

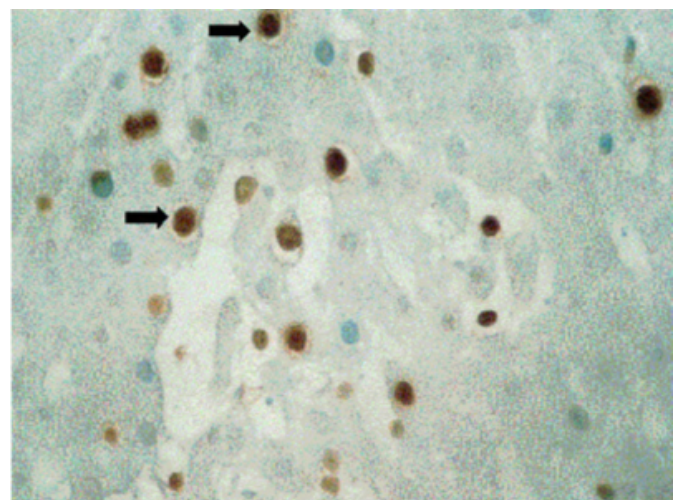


Figure 4

Figure 2a: Apoptotic hepatocytes (brown stained cytoplasm) in periportal area in 85% hepatectomy group (x460).

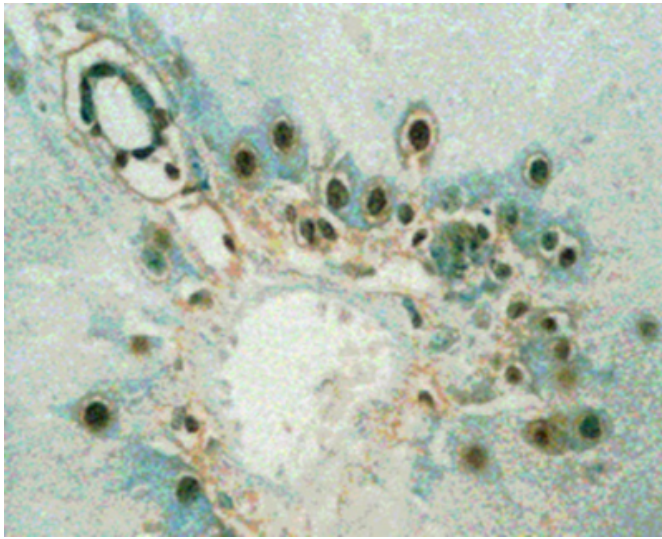
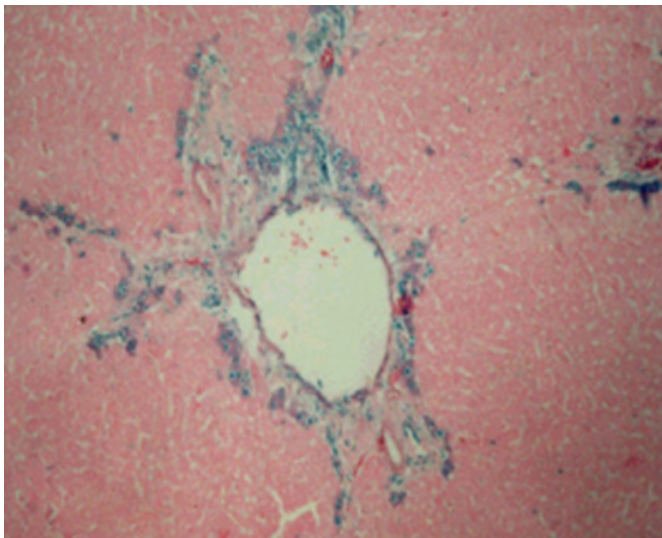


Figure 5

Figure 2b: Blue stained necrotic hepatocytes in periportal area (85% hepatectomy group) (x115).



DISCUSSION

In this study, 85% hepatectomy model chosen because of a few rats to survive without metabolic support and 70% hepatectomy characterizes the normal physiologic response. Pannis et al. demonstrated that in rats the 85% hepatectomy model is a transition between a high rate of survival (more than 80%) and 0% survival because the remnant after 85% hepatectomy is one half of that after 70% hepatectomy¹.

Apoptosis could be detected by some methods, such as TUNEL (terminal deoxynucleotidyl transferase-mediated

dUTP nickend labeling), ISEL (in situ nickend labeling) and flow cytometry⁸. In this study, we used the immunocytochemical method using ApopTag peroxidase kit, (Intergen company). This method detects apoptotic cells via DNA fragmentation and also allows the early detection of apoptosis before characteristic microscopic findings appear.

Apoptosis, or programmed cell death, and the elimination of apoptotic cells are crucial factors in the maintenance of liver health. Apoptosis allows hepatocytes to die without provoking a potentially harmful inflammatory response. Viral and autoimmune hepatitis, cholestatic diseases, and metabolic disorders are often associated with enhanced hepatocyte apoptosis. Apoptosis also plays a role in transplantation-associated liver damage, both in ischemia/reperfusion injury and graft rejection^{3,4}. On the other hand, Gujral et al. demonstrated that after various times of warm hepatic ischemia, cell injury during reperfusion occurred through oncotic necrosis⁹. The role of apoptosis in various liver diseases and the mechanisms by which apoptosis occurs in the liver may provide insight into these diseases and suggest possible treatment modalities⁴.

In the case of necrosis, cytosolic constituents that spill into extracellular space through damaged plasma membrane may provoke inflammatory response; during apoptosis these products are safely isolated by membranes and then are consumed by macrophages. Necrosis constitutes inflammatory response⁴. Apoptosis allows hepatocytes to die without provoking a potentially harmful inflammatory response in contrast to necrosis^{4,5}.

After extensive/lethal hepatectomy the remnant hepatocytes immediately produce acute phase proteins (C-reactive protein, α 1-acid glycoprotein, fibrinogen and α 2-macroglobulin) and Kupffer cells are significantly activated. Kupffer cells activation and sinusoidal endothelial cell injury accompanied by a significant increase in production of TNF- α , lead to extensive induction of apoptosis in hepatocyte but the mechanism is still unclear^{2,3}. At the same time, apoptosis is tightly controlled and regulated via several mechanisms, including Fas/Fas ligand interactions, the effects of cytokines such transforming growth factor beta (TGF-beta), and the influence of pro- and antiapoptotic mitochondria-associated proteins of the B-cell lymphoma-2 (Bcl-2) family. Efficient elimination of apoptotic cells in the liver relies on Kupffer and endothelial cell functions.

In Hasegawa's study, authors showed that, apoptosis is the

major determinant of liver failure after lethal hepatectomy. Similarly, Nagano et al concluded that after extensive hepatectomy, apoptosis signal transduction appears to predominate over antiapoptosis signal transduction. In these studies apoptotic and necrotic cell numbers were not counted together^{2,3}. In Morita's study, authors used cDNA microarray analysis to compare clearly differentiated rat partial hepatectomy models and they concluded that fatal hepatic failure after excessive hepatectomy was characterized by increased apoptosis and diminished liver regeneration¹⁰.

On the other hand, clinical significance of the apoptosis in remnant liver is still unclear. Does inhibition of apoptosis prevent liver failure after lethal/extensive hepatectomy? In our opinion, apoptosis constitute less inflammatory response than necrosis and inhibition of apoptosis may constitute much necrosis and much inflammation. This deserves to be an interesting research topic.

We concluded that lethal hepatic failure after excessive hepatectomy was characterized by increased apoptosis.

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