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Fas and Fas-ligand Expression in Hepatocytes during Regeneration after Partial Hepatectomy in Rats

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Abstract The regenerative response after partial hepatectomy stops suddenly when liver mass has been restored. In this regulation, arrest of hepatocyte proliferation in addition to elimination of excess hepatocytes would appear to be necessary. To determine whether excess hepatocytes are eliminated by Fas-mediated apoptosis, we examined expression of Fas and Fas-ligand in hepatocytes using immunohistochemical staining after 70% partial hepatectomy. In addition, apoptotic hepatocytes were identified using *in situ* DNA end-labeling. Fas-positive, Fas-ligand-positive, and apoptotic hepatocytes were detected only rarely in the uninjured liver. Fas-positive hepatocytes increased somewhat on day 3 following hepatectomy and significantly on days 4 to 6, then decreasing on day 8. Fas-ligand-positive hepatocytes significantly increased on days 3 to 5 following hepatectomy, and then decreased on day 6. Apoptotic hepatocytes increased somewhat on day 4, markedly on day 6, and then decreased on day 14. According to these findings, apoptosis began to occur in hepatocytes 1 day after initial expression of Fas and its ligand. These apoptotic changes may be explained by the consideration that some abruptly regenerated hepatocytes may not be normal and eventually have a short-life time or actively eliminated by some surveillance system. The earlier and simultaneous expression on hepatocytes of Fas and its ligand may indicate paracrine or autocrine mechanisms of cell elimination executed by the hepatocytes themselves.

Key words : Fas, Fas-ligand, Apoptosis, Hepatocyte, Liver regeneration

Introduction

The ability of the liver to regenerate rapidly and precisely presents one of the most fascinating and mystifying problems in science. Regenerative halts abruptly following insults such as partial hepatectomy (PH) when liver mass has been restored, and no significant overshoot is seen despite the rapid proliferation that occurs during regeneration. Clearly, important negative regulatory influences, as well as positive ones are at work. Both control of hepatocyte proliferation and the elimination of excess hepatocytes are likely to be involved, but the mechanisms that eliminate excess hepatocytes during liver regeneration still are unknown.

A few specific growth-inhibitory factors have been identified[1]. Transforming growth factor beta-1 (TGF-β1) appears to be a negative regulator of hepatocyte proliferation. In hepatocytes, TGF-β1 inhibits DNA synthesis[2, 3]. Activin A, a member of the TGF-β gene superfamily, has been shown to markedly inhibit the increasing DNA synthesis induced by epidermal growth factor (EGF) in cultured primary hepatocytes[4,5]. Interleukin (IL) -1β[6,7], IL-6,[6,7], interferon (INF) -α[8], and hepatocyte proliferation inhibitor (HPF)[9] also inhibit the liver regeneration.

Two form of cell death have been described in vertebrate tissues. Necrosis, a pathologic response involving a dramatic increase in cell volume that ultimately leads to lysis, is a form of cell death induced by exposure to noxious compounds or other insults[10]. Apoptosis, on the other hand, is a more subtle process which commonly occurs when cell death is physiologically determined. These two forms of cell death are clearly distinguishable morphologically[11]. Fas, a 319-amino acid cell-surface protein of molecular weight 45 kDa, is a member of the tumor necrosis factor (TNF) receptor superfamily, which includes TNF receptors type I and II, the low affinity nerve growth factor receptor, CD40, CD27, CD30, and OX40. Fas is expressed at high levels in various tissues[13, 14]. Recently the cDNA for the ligand to Fas also has been cloned[15,16]. The Fas-ligand (Fas-L) is a type II integral membrane protein homologous to tumor necrosis factor that has been detected on cytotoxic T lymphocytes (CTL) and demonstrated to mediate apoptotic cell death on Fas-expressing T cells[17]. Fas-L also has been reported to mediate autocrine T-cell suicide[18]. Ogasawara et al[19]. have demonstrated that anti-Fas antibody injected i.p. induces massive liver damage in mice having the Fas antigen on their hepatocytes; this antibody showed no effect on the livers of *lpr* mice that had genetically lost the expression of the Fas antigen. Anti-Fas antibody reportedly can induce apoptosis of mouse hepatocytes in primary culture[20]. Fas-mediated apoptosis also has been observed in the human liver during allograft rejection[21] and in the course of chronic hepatitis C[22]. The possibility that an excess of tissue and hepatic DNA content could be rapidly eliminated by apoptotic cell death following withdrawal of the growth stimulus has been reported[23,24]. Recently, Galle et al[25]. have detected high levels of hepatocytic CD95 ligand (Fas-L) mRNA expression in hepatocytes in livers with ongoing damage but not in normal livers. These studies promoted us to examine whether elimination of excess hepatocytes when regeneration following PH is nearing completion is a result of Fas-mediated apoptosis.

We examined expression of Fas and Fas-L in rat hepatocytes after PH using immunohistochemical staining and identified apoptotic hepatocytes using *in situ* DNA end labeling.

Materials and Methods

Animals

Specific pathogen-free male Donryu rats, weighing 250 to 300g, were used. All animals were housed under controlled temperature and humidity conditions, with a 12-hr alternating light/dark cycle, and free access to food and water. The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at the Yamaguchi University School of Medicine, and carried out according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine as well as Law No. 105 and Notification No. 6 of the Japanese Government.

Surgical Procedure

Seventy-percent PH was done on 27 rats under ether anesthesia according to the method described by Higgins and Anderson[26]. The median and left lateral lobes were removed, while the others were disturbed minimally and left *in situ*.

Specimens

To evaluate expression of Fas and Fas-L, identify apoptotic hepatocytes, and assess liver regeneration, a total of 30 rats were killed at 1 , 2, 3, 4, 5, 6, 7, 8, and 14days after operation and the liver remnants were removed. The livers of untreated rats (n=3) were used as controls. Aliquots of liver tissue were fixed in 10% formalin for subsequent immunohistochemical analysis and hematoxylin and eosin (HE) staining.

Calculation of the mitotic index

For each HE-stained post-PH hepatic specimen, hepatocytes in mitosis were counted. The mitotic index (MI) was taken as the number of hepatocytes in mitosis per 1000 hepatocytes. All sections were assessed by two of the authors who were unaware of the experimental group examined.

Antibodies

Immunohistochemistry was performed using polyclonal antibodies to Fas (P4) and Fas ligand (P5). The specificity of P4 and P5 has been reported previously[27].

Immunohistochemical Staining of Fas Antigen and Fas-L

All paraffin blocks containing liver specimens were sectioned at a 3-μm thickness. Sections were dewaxed and stained with P4 and P5 using a labeled streptavidin biotin (LSAB) kit (DAKO, Carpinteria, CA) as described below. Deparaffinized and rehydrated sections were immersed in 3% H_2O_2 to block endogenous peroxidase, and were preincubated with 10% normal goat serum in phosphate-buffered saline (PBS) for 30 min at room temperature to block nonspecific binding of antibodies. Then the sections were allowed to react with the primary antibodies diluted with PBS (1 : 500) for 1 hr at room temperature in a humidified atmosphere. After washing in PBS 3 times for 15 min per wash, sections were incubated with goat anti-rabbit secondary antibody for 1 hr at room temperature, and washed again as described above. The sections were incubated with peroxidase conjugated streptavidin. Sites of horseradish peroxidase binding were visualized using 3,3'-diaminobenzidine and H_2O_2. As a control, some sections were exposed to normal rabbit serum in place of the specific antibody, and some were incubated with primary antibody in the presence of an excess amount of the synthetic peptide used to raise the antibody at a 500-fold molar ratio. Samples then were counterstained with hematoxylin and mounted. Fas or Fas-L positive hepatocytes were counted in 20 high-power field (HPF) (magnification, × 200). All sections were read by two of the authors who were unaware of the experimental group.

Apoptosis Analysis

Apoptosis was detected by using a nonisotopic in situ end-labeling (ISEL) technique, with a commercially available kit (Apotag™ ; Oncor, Gaithersburg, MD) which was applied to paraffin-embedded sections cut from the same blocks used for morphometric analysis[28-30]. The kit manufactur's protocol was followed. Briefly, in this ISEL technique, digoxigenin-labeled nucleotides residues were added catalytically to the fragmented DNA (3'-OH ends) by the enzyme terminal deoxynucleotidyl transferase. The added residues then were immunostained by anti-digoxigenin-peroxidase antibody. The localized peroxidase enzyme was then revealed by routine diaminobenzidine staining (Sigma Chemical Co., St. Louis, MO). The sections were counterstained with hematoxylin. Apoptotic cells were counted in 20 HPF (magnification ; × 200). All sections were read by two authors who were not aware of the experimental group.

Randomization and Statistical Analysis

The rats were assigned randomly to one of the nine groups at the time of killing by blind selection from a box of a card bearing the group number and day of killing. When a rat was excluded for any reason, the same operation was performed immediately on a replacement rat. All results are expressed as the mean ± standard error (SE). Statistical analyses were performed using three-way analysis of variance (ANOVA). Fisher's protected least-significance difference test was used for multiple comparisons. Significance was accepted at the 5% level.

Results

Three rats were excluded from these experiments because of portal vein injury, ischemia of the remnant liver or other mishap.

Mitotic Index

The highest values of MI in remnant liver were observed 1 day after PH. Beginning from 2 days after operation, MI decreased (Table 1).

Fas-positive Hepatocytes

A few Fas-positive (Fas^+) hepatocytes were detected in uninjured control liver (Fig. 1a). Fas^+ hepatocytes increased on day 3 following PH, and significantly increased on days 4 to day 6 ($P < .01$) (Table 1). On days 8 and 14 (Fig. 1c), numbers of Fas^+ he-

Table 1. Change of MI, Fas-positive, Fas-ligand-positive, and apoptotic hepatocytes during liver regeneration.

	MI	Fas	Fas-ligand	Apoptotic cell
Control	6.0	2.0	1.0	0.3
Day 1	68.0*	2.0	1.0	1.0
Day 2	42.3*	2.0	1.0	0.3
Day 3	23.0#	5.0	5.0#	1.0
Day 4	15.0	20.3*	6.3*	3.3
Day 5	8.7	19.0*	5.0#	3.0
Day 6	5.7	19.7*	1.0	19.0#
Day 7	5.3	4.3	1.0	5.3#
Day 8	5.7	2.3	1.0	6.0#
Day 14	5.3	2.0	1.0	3.0

NOTE. Values are given as mean ± SE (MI ; ± 2.39, Fas ; ± 0.78, Fas-lagand ; ± 0.56 ; Apoptotic cells, ± 0.76). *: $p < 0.01$ vs. control, # $p < 0.05$ vs. control.

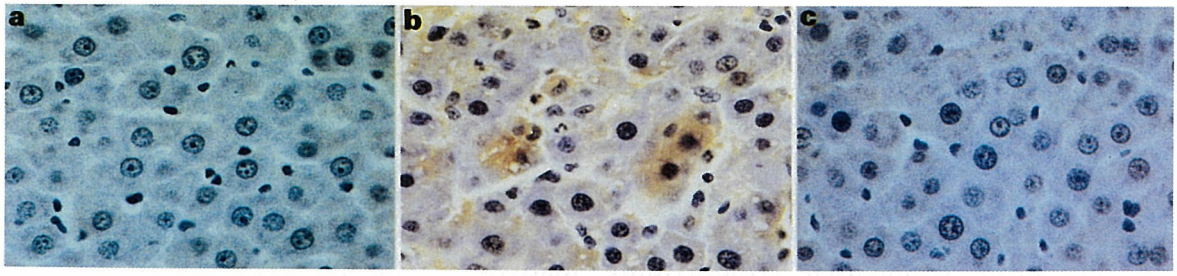

Fig. 1. Immunohistochemical demonstration of Fas^+ apoptotic hepatocytes during liver regeneration after 70% hepatectomy. Three-micrometer rat liver sections were stained using a labeled streptavidin biotin kit to identify Fas. Only a few Fas^+ hepatocytes not seen in this photomicrograph were detected in uninjured liver (control ; a). Fas^+ hepatocytes increased on day 4 after hepatectomy (b). Fas^+ hepatocytes decreased on day 14 (not seen ; c). Original magnification, ×400.

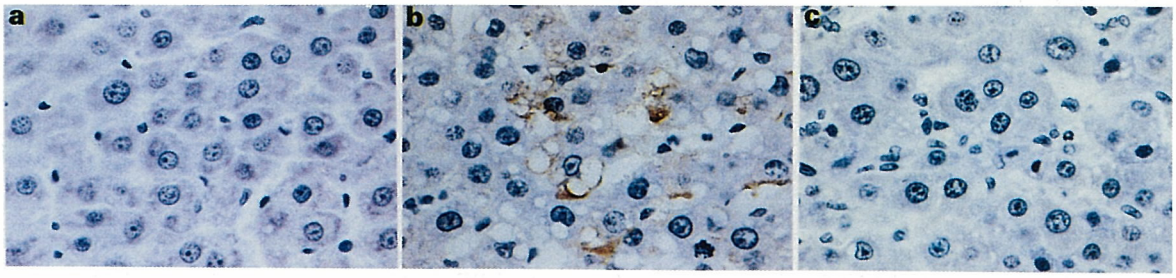

Fig. 2. Immunohistochemical demonstration of $Fas\text{-}L^+$ hepatocytes during liver regeneration after 70% hepatectomy. The stain was performed by same method as for Fas. Few $Fas\text{-}L^+$ hepatocytes (not shown) were detected in control liver (a). $Fas\text{-}L^+$ hepatocytes increased on day 4 after hepatectomy (b). $Fas\text{-}L^+$ hepatocytes (not shown) decreased on day 14 (c). Original magnification, ×400.

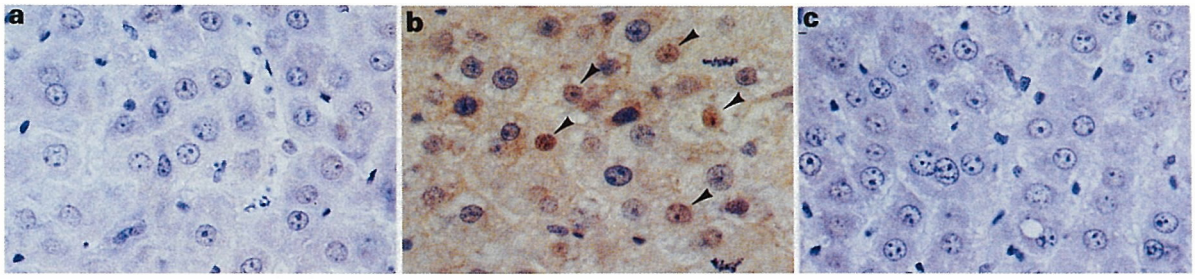

Fig. 3. Immunohistochemical demonstration of apoptotic hepatocytes during liver regeneration after 70% hepatectomy. Three-micrometer rat liver sections were stained using a nonisotopic in situ end-labeling (ISEL) technique. Few apoptotic hepatocytes (not shown) were detected in control liver (a). Fas-L^+ hepatocytes (arrows) increased on day 6 after hepatectomy (b). Apoptotic hepatocytes (not shown) decreased on day 14 after (c). Original magnification, ×400.

patocytes were identical to those in controls. Figure 1b shows Fas^+ hepatocytes on day 4.

Fas-L-Positive Hepatocytes

Few Fas-L positive (Fas-L^+) hepatocytes were detected in control liver (Fig. 2a). Fas-L^+ hepatocytes significantly increased on days 3 to 5 following PH ($P < .05$) (Table 1). On days 6, 7, 8, and 14 (Fig. 2c), the number of Fas-L^+ hepatocytes were identical to those in controls. Figure 2b shows the Fas-L^+ hepatocytes on day 4.

Apoptotic Hepatocytes

Few apoptotic hepatocytes were detected in control liver (Fig. 3a). Apoptotic hepatocytes increased somewhat on day 4, increased markedly on day 6 ($P < .01$), and increased significantly even on days 7 to 8 following PH ($P < .05$) (Table 1). On day 14 (Fig. 3c), the number of apoptotic hepatocytes still was increased compared to control liver, but not significantly. Figure 3 b shows apoptotic hepatocytes on day 6.

Time Course of MI, Fas^+, Fas-L^+, and Apoptotic Hepatocytes after PH

Figure 4 shows the time course of MI and appearance of Fas^+, Fas-L^+, and apoptotic hepatocytes after PH. The peaks of MI and appearance of Fas^+, Fas-L^+, and apoptotic hepatocytes were on day 1, on days 4 to 6, on days 3 to 5, and on day 6, respectively. Apoptosis occurred following Fas and Fas-L expression on hepatocytes after PH.

Discussion

The typical course of liver regeneration after PH in rat is as follows[31] : a semi-synchronous wave of DNA synthesis begins at approximately 12 to 15 hr, and peaks by 20 to 24 hr, with the liver nearly doubling in size at 48 hr. By 7 to 10 days, growth abruptly ceases, with the original mass of the liver entirely restored. Our mitotic index data were consistent with this sequence. Although the mechanisms controlling cessation of hepatocyte proliferation have been explored recently, only a few specific growth inhibitory factors have been identified.1 Both proliferation arrest and excess-hepatocyte elimination of excessive hepatocytes are likely to be involved. The increase in number of hepatocytes may be complete within 72 hr after PH, and elimination of excess hepatocytes may follow or occur simultaneously.

Apoptosis is a type of cell death that serves to eliminate superfluous or unwanted cells.[32,33] TGF-β1[34-36] and activin A[37] have been reported to induce apoptosis in hepatocytes. However, the intracellular pathways involved in apoptosis are not well understood. Recently, expression of apoptosis-associated genes such as Bcl-2, Bcl-x, Bax, Bak, and Bad has been reported to change during liver regeneration. Kren et al[38]. have reported that transcripts for both Bcl-x and Bcl-2 exhibited peaks at 6 hr and bax transcript exhibited peaks at 18 and 72 hr after PH in rats. Tzung et al[39]. also demonstrated the possibility that Bcl-2 family members such as

Bcl-x, Bak, Bad, and Bax may relate to cell cycle control and apoptosis during liver regeneration in mice. These reports suggest that apoptosis of hepatocytes occurs during liver regeneration.

Fas antigen mediates apoptosis by the interaction with Fas-L[17]. Ogasawara et al[19]. demonstrated that intraperitoneal administration of anti-Fas antibody induced massive liver damage in mice with the Fas antigen on their hepatocytes. They also showed that antibody administration did not induce an effect on the liver of *lpr* mice, which have generally lost expression of the Fas antigen. Fas expression in mouse[19,20], rat[40], and human[21-25] hepatocytes has been reported. Fas-L mRNA also is expressed in damaged hepatocytes[25]. We therefore hypothesized that the elimination of excess hepatocytes during liver regeneration may be result from Fas-mediated apoptosis.

The Fas/CD95 pathway constitutes a major mechanism of T cell-mediated cytotoxicity[40-44]. Recently, expression of Fas-L has been shown for mononuclear cells infiltrating the liver in patients with hepatitis C[45]. Therefore, activated T-killer cells may kill HBV-infected hepatocytes using the Fas/CD95 system. However, we observed only a few infiltrating lymphocyte in any liver section after PH (data not shown). We reported that IL-2, which activates CTL[46], did not affect liver regeneration after hepatectomy[47]. These findings indicate little involvement of inflammatory mediators, including CTL, in this process. Galle at al[25]. have reported that the CD95 ligand (Fas-L) was expressed at high levels in hepatocytes affected by human alcoholic hepatitis. Since both of ligand and receptor mRNA coexisted, they speculated that in alcoholic liver damage death of hepatocytes might occur by fratricide and paracrine or autocrine mechanisms mediated by the hepatocytes themselves. In the present study, Fas^+, $Fas\text{-}L^+$, and apoptotic hepatocytes were rarely detected in the untreated liver. Fas^+ hepatocytes increased somewhat on day 3 following PH, increased significantly on days 4 to 6, and then decreased on day 8. $Fas\text{-}L^+$ hepatocytes increased significantly on days 3 to 5 following PH, and then decreased on day 6.

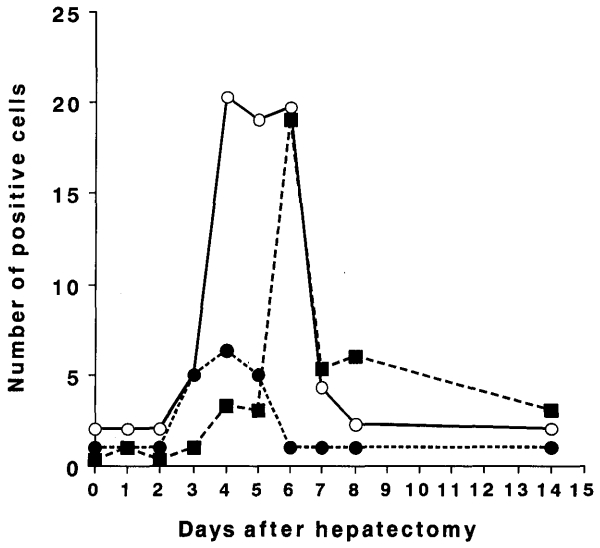

Fig. 4. Time course of Fas^+, $Fas\text{-}L^+$, and apoptotic hepatocytes after partial hepatectomy. Values are expressed as the mean. ○—○ ; Fas^+ hepatocytes, ●-----● ; $Fas\text{-}L^+$ hepatocytes, ■---■ ; apoptotic hepatocytes. Each positive hepatocytes were counted in 20 high-power field (HPF) (magnification, ×200).

Apoptotic hepatocytes increased somewhat on day 4, increased markedly on day 6, and then decreased on day 14. These apoptotic changes may be explained by the consideration that some abruptly regenerated hepatocytes may not be normal and eventually have a short-life time or actively eliminated by some surveillance system. Furthermore, the apoptosis may be autocrine in its triggering mechanism because both Fas and Fas-L were expressed in hepatocytes. Fas^+ and $Fas\text{-}L^+$ hepatocytes began to be seen about 1 day before apoptosis (Fig. 4). Since apoptotic cells are rapidly cleared by phagocytosis in vivo[48], apoptotic hepatocytes most likely were eliminated by Kupffer cells on days 4 and 5 after PH. However, the number of apoptotic hepatocytes probably became too large to be eliminated rapidly by phagocytosis from days 6 to 8.

In conclusion, excess hepatocytes present in late stage of liver regeneration after PH may be eliminated by Fas-mediated apoptosis controlled by hepatocyte-mediated mechanisms.

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