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Toxic Bile Salts-Induced Apoptosis of Hepatocytes in Biliary Obstruction Involves Fas-independent Pathway

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Abstract. Cholestatic liver injury results from the accumulation of toxic bile salts within the liver. The aim of the present study is to elucidate the changes in expression and cellular localization of apoptosis related proteins in the liver of bile duct-ligated (BDL) rat. Extrahepatic cholestasis was induced by double ligation of the common bile duct and cut between the ligatures. Animals were sacrificed at day 3 and at week 1, 2, 4, 6, and 8 after BDL. The number of TUNEL positive cells was increased significantly after 3 days of BDL, decreased over 2 weeks and remained constant thereafter. Fas expression was not changed and activation of caspase 8 did not occur. Fas immunoreactivity was exclusively observed in the cytoplasm of hepatocytes, indicating that Fas expressed in rat hepatocytes is a soluble form. Hepatocyte apoptosis was associated with Bax expression, which showed a peak at day 3 and decreased over time gradually. Immunostaining of Bax was observed in hepatocytes and bile duct epithelial cells (BEC) of control and BDL rats. Bcl-2 was increased over time in BDL rats. These results suggest that apoptosis of hepatocytes in BDL rats is independent of Fas and controlled by Bax expression.

Keywords: Bile duct ligation, Apoptosis, Hepatocyte, Fas, Bax.

INTRODUCTION

Cholestasis is a common pathophysiological process in many human liver diseases leading to the accumulation of toxic bile salts within the liver. Persistence of cholestasis is associated with acute and chronic liver failure, leading to biliary fibrosis, cirrhosis and cancer. Hepatocellular damage in cholestasis is related to the retention of toxic bile salts, which are known to induce apoptosis. Although the mechanism of bile salts-mediated apoptosis is not yet completely understood, two pathways have been suggested: activation of the Fas death receptor, and translocation of Bax to the mitochondria (Miyoshi *et al.*, 1999; Rodrigues *et al.*, 1998; Faubion *et al.*, 1999; Galle *et al.*, 1995; Ogasawara *et al.*, 1993; Rodrigues *et al.*, 1999).

Fas/APO-1 is a 45 kDa cell surface receptor protein belonging to the TNF receptor family. When activated by Fas ligand, Fas receptor trimerizes and results in the activation of caspase 8, which in turn activates downstream caspases including caspase 3, an executioner caspase leading to cell death (Li *et al.*, 1998; Gross *et al.*, 1999b). Hepatocytes express Fas and are therefore potential targets for Fas ligand-mediated injury. Injection of agonistic anti-Fas antibody into mice induces massive apoptosis of hepatocytes, but Fas-deficient lpr mice are completely resistant to this (Miyoshi *et al.*, 1999; Ogasawara *et al.*, 1993). In bile duct-ligated (BDL) mice, hepatocyte apoptosis occurs predominantly by Fas-dependent pathway (Miyoshi *et al.*, 1999). *In vitro* experiments also demonstrate that apoptosis of mouse hepatocytes induced by toxic bile salts involves ligand-independent oligomerization of Fas (Faubion *et al.*, 1999). The *fas* gene encodes mainly two isoforms, the membrane bound Fas (mFas) and soluble Fas (sFas). The former is translated from the Fas full-length mRNA and the latter from alternatively spliced mRNA lacking the transmembrane domain. sFas may have an advantage for survival because it may inhibit Fas-medi-

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Abbreviations: BDL, bile duct ligation; BEC, bile duct epithelial cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; mFas, membrane bound Fas; sFas, soluble Fas; RT-PCR, reverse transcriptase-polymerase chain reaction.

ated apoptosis by blocking FasFas ligand interactions (Cheng *et al.*, 1994; Krams *et al.*, 1998). It may even provide a mechanism for tumor cells to escape immune surveillance, thus favoring carcinogenesis, including hepatocellular carcinoma and cholangiocarcinoma (Sacco *et al.*, 2000; Que *et al.*, 1999). In the present study we investigated the molecular mechanism of hepatocellular apoptosis in BDL rats especially in terms of the involvement of Fas.

MATERIALS AND METHODS

Animal Treatment and Biliary Obstruction

Specific pathogen-free male Sprague-Dawley rats (200~220 g) were obtained from Daehan Laboratory Animal Research and Co. (Choongbuk, Korea) and allowed free access to standard chow and tap water. They were kept in temperature controlled and filter-sterilized animal quarters under a 12 h/12 h light-dark cycle. They were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.), and the common bile duct was double-ligated and cut between the ligatures. Animals were killed at day 3 and at weeks 1, 2, 4, 6 and 8 after BDL. Sham-operated rats served as controls. Unoperated animals were used as day 0 controls.

Antibodies

Mouse monoclonal antibodies against Bcl-2 and Bax and rabbit polyclonal antibody against Fas were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against p53 (clone PAb 240) and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Neomarkers (Fremont, CA) and Zymed (San Francisco, CA), respectively. Goat anti-mouse IgG was a product of Boehringer Mannheim (Mannheim, Germany).

TUNEL Staining

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using the Boehringer *in situ* death detection kit (Mannheim, Germany). Formalin-fixed liver tissues were dehydrated through increasing concentrations of ethanol and embedded in paraffin wax. Sections (4 μm) were deparaffinized in xylene. After the sections had been rehydrated in phosphate buffered saline, they were incubated with pepsin solution for 10 min at room temperature and assayed for TUNEL analysis, as recommended by the supplier. The number of positive cells was counted in 20 random fields for each specimen using a confocal microscope (Olympus, ×200).

Western Blot Analysis

Isolated liver tissues were homogenized in RIPA buffer. After the homogenates were centrifuged at 600 ×g for 15 min at 4°C, the supernatants were collected and the protein concentration was measured. For detection of Fas, Bcl-2 and Bax, tissue extract (70 μg) were separated onto 12% acrylamide gels and then transferred on nitrocellulose membrane. After incubation with primary antibodies, the membranes were washed and incubated with secondary antibodies (1:1500) for 1 h at room temperature. Proteins were visualized using electrochemiluminescence Western Blotting Detection System (Amersham, UK).

Immunohistochemical Staining

The preparation of sections was the same as described for the TUNEL assay. The sections were then subjected to antigen retrieval by autoclaving in 0.01 M citrate buffer (pH 6.0). Proteins were detected by avidin-biotin complex staining. Tissue sections were incubated overnight at 4°C with primary antibodies of 1:50 dilution of anti-Fas, anti-Bax and anti-Bcl-2 in primary antibody diluents. Alternate sections were treated with normal rabbit serum or antibody diluents as controls. The peroxidase activity was detected with 3-amino-9-ethylcarbazole and counterstained with hematoxylin.

Reverse-Transcriptase PCR (RT-PCR)

Total cellular RNA was extracted using RNeasy Mini kit (Qiagen Inc., CA). Two mg of total cellular RNA per sample was synthesized to the first strand cDNA using AMV reverse transcriptase XL and oligo dT-adaptor primers (Takara Biomedicals, Japan). PCR amplification was carried out using RNA PCR kit (Takara Biomedicals, Japan). Amplification for mFas and sFas was performed with the following profile: an initial denaturation at 94°C for 2 min followed by 32 and 40 cycles of denaturation (94°C, 1 min), annealing (51°C, 1 min), and elongation (72°C, 1.5 min). Cycling condition for GAPDH amplification was identical except that the annealing temperature was set to 56°C and amplification cycles were reduced to 30. Primer pairs for mFas (PCR product of 142 bp) and sFas (PCR product of 149 bp) of Rat were synthesized according to Kimura *et al.* (1994). GAPDH primer sequences were GAPDH sense primer, 5'-CCCCTTCATTGACCTCAACTAC-3', and GAPDH antisense primer, 5'-CATGGTGGTGAAGACGCCAG-3'. Twelve μl (6 μl in the case of GAPDH) of each PCR was analyzed by electrophoresis in 1.8% agarose gels.

A 1 Kb Plus DNA ladder served as a molecular size marker (Gibco BRL, NY).

RESULTS

Apoptotic Death of Hepatocytes

We analyzed the apoptotic death of hepatocytes in BDL rats using TUNEL staining. TUNEL positive cells were readily identified by nuclear condensation. There were no differences in the number of apoptotic cells between control and sham-operated animals (data not shown). In BDL rats, the number of TUNEL positive cells increased five-fold by 3 days, decreased until 2 weeks and remained constant thereafter (Table 1).

Table 1. Hepatocellular apoptosis in liver tissues of BDL rats

Days after BDL	Apoptotic cells
Control	1.4±0.5
3 days	10.1±2.4*
1 week	7.7±0.6**
2 weeks	4.7±1.6
4 weeks	5.4±1.5
6 weeks	5.4±1.1
8 weeks	5.2±1.7

Apoptosis was detected by the TUNEL assay as described in Materials and Methods. TUNEL positive cells were detected by confocal microscope (excitation: 490 nm; emission: 520 nm). Data are expressed as means±SEM of 3 separate experiments. *P < 0.02, **P < 0.001.

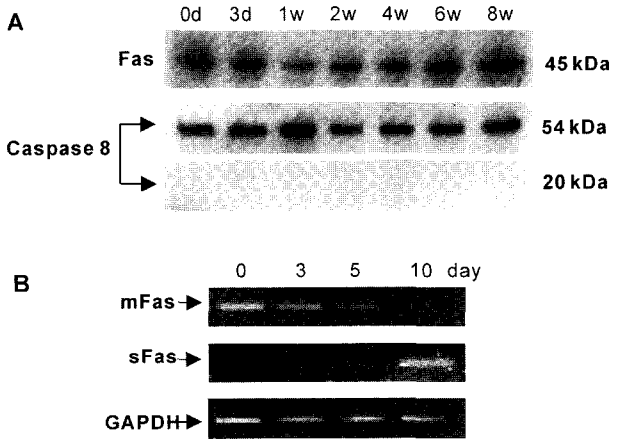

Fig. 1. (A) Western blot analysis of Fas and caspase 8. Liver tissues were obtained from control and BDL rats. Seventy μg proteins were separated by 12% acrylamide gels, transferred to nitrocellulose membrane and probed with antibodies recognizing Fas (45 kDa), pro- (54 kDa) and active caspase 8 (20 kDa). (B) RT-PCR analysis of Fas transcript. The expression of mFas and sFas were determined by RT-PCR with primers that are specific for each gene as described in 'Materials and Methods'. Representative results obtained from at least three independent experiments were shown.

Involvement of Fas in Toxic Bile Salt-induced Apoptosis

To investigate whether Fas-death pathway is functional in BDL rats, we examined the expression of Fas. Fas expression was slightly increased until 8 weeks of BDL as shown in Western blot analysis. However, activation of caspase 8 did not occur (Fig. 1A). To elucidate cellular localization of the protein, we performed immunohistochemical analysis. Immunoreactivity of Fas protein was mainly observed in the cytoplasm of hepatocytes but not in bile duct epithelial cells (BEC). In control rats, Fas protein appeared as conspicuous punctuate structure associated with cytosolic protein aggregates and gathered near cytoplasmic membrane (Fig. 3A). In BDL rats staining pattern of Fas showed granular structures that were evenly dispersed throughout the cytoplasmic compartment. We could not find Fas staining in cytoplasmic membrane compartment (Fig. 3B, C). To confirm the role of Fas in BDL rats, we performed RT-PCR from liver tissues at day 3, 5 and 10 after BDL. As shown in Fig.1B, mFas was decreased until 5 days before it was disappeared by 10 days after BDL. By contrast, the transcript for sFas, lacking the trans-

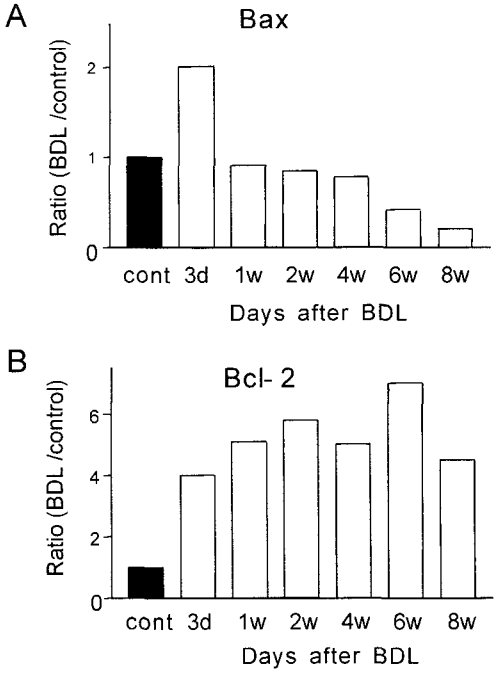

Fig. 2. The density of the bands obtained from Western blot analysis of Bax (A) and Bcl-2 (B) were analyzed using an image analyzing system. Each value represents the ratio of the density from BDL to control rats in arbitrary densitometric units.

membrane domain, appeared at 5 day and was evident by ten days (Fig. 1B).

Involvement of Bcl-2 Family Protein in Toxic Bile Salt-induced Apoptosis

We next examined whether Bax-mediated apoptosis was implicated. Bax protein showed a significant increase after BDL for 3 days, thereafter decreased over time. At 8 week Bax expression was very weak (Fig. 2A). Bax protein was localized in the cytoplasm of hepatocytes and BEC and appeared as dot structures. Bax was detectable not only in hepatocytes, as previously reported (Ogasawara *et al.*, 1993), but also in BEC (Fig. 3D~F). Parallel to the results of Western blot analysis, staining intensity of Bax was decreased in hepatocytes and BEC, and after 8 weeks it was very fainted (Fig. 3F). Bcl-2 expression was increased throughout 8 weeks after BDL as can be seen in Western blot and immunohistochemical analysis (Fig. 2B, Fig. 3G~I).

DISCUSSION

Liver injury by biliary obstruction leads to inflammation and death of the hepatocytes. If hepatic injury and cell death are progressive, it results in liver fibrosis, cirrhosis and cancer (Kountouras *et al.*, 1984). It is now generally recognized that toxic bile salts induce apoptosis at the low concentrations that are typically observed during cholestasis. Recent investigations have focused

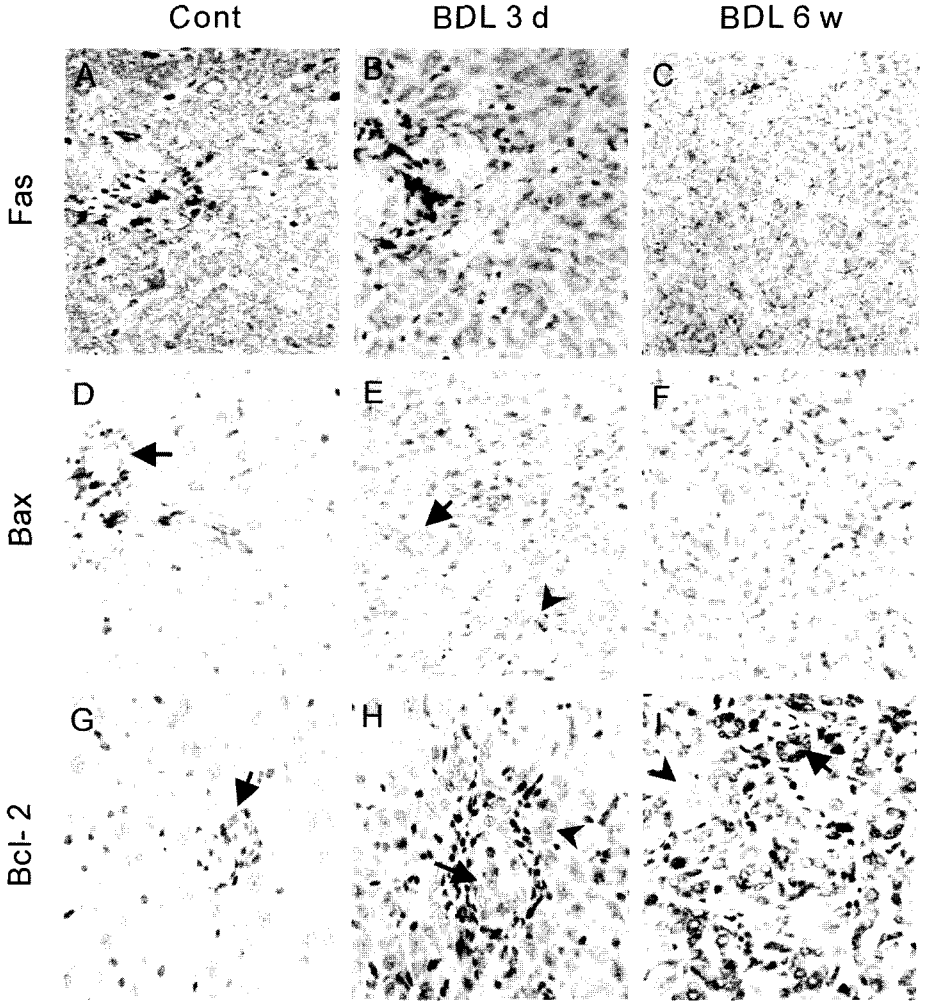

Fig. 3. Immunohistochemical localization of Fas, Bax and Bcl-2 protein. Liver tissues were obtained from control and BDL rats, and incubated with anti-Fas, anti-Bax and anti-Bcl-2 antibodies. Immunoreactivity of Fas protein was mainly observed in the cytoplasm of hepatocytes (➤) but not in BEC (►) (A-C). Bax protein was localized in the cytoplasm of hepatocytes and BEC and appeared as dot structures. Bax was detectable not only in hepatocytes but also in BEC (D-F). Bcl-2 expression was increased throughout 8 weeks after BDL (G-I).

on the elucidation of the cellular and molecular mechanisms of apoptosis by toxic bile salts.

In our previous study, we found that p53 plays an important role in toxic bile salts induced hepatocyte apoptosis in BDL rats (Oh *et al.*, 2003). According to our immunohistochemical study, p53 was accumulated in the nucleus soon after BDL whereas cytoplasmic sequestration of p53 was observed in prolonged BDL rats. p53 is a nuclear protein, and nuclear localization is essential for acting as a transcriptional factor. However cytoplasmic sequestration of wild-type p53 protein is observed in cases of mutagenesis, viral oncogenesis, and some types of cancer, which is known to be functionally inactive (McKenzie *et al.*, 1999; Moll *et al.*, 1996). Wild-type p53 plays a pivotal role in cell cycle checkpoint after DNA damage, and induces G1 arrest or apoptosis. One transcriptional target of p53 that may be important for apoptosis is Bax. Overexpression of p53 increases Bax expression in several cell types and induces apoptosis (Selvakumaran *et al.*, 1994; Han *et al.*, 1996; Xiang *et al.*, 1998). Although the mechanisms by which Bax promotes apoptosis are not thoroughly understood, several studies provide convincing evidence that Bax directly induces mitochondrial permeability transition and Cyt *c* release, by interacting with the permeability transition pores (Narita *et al.*, 1998; Jürgensmeier *et al.*, 1998). Bcl-2, which can inhibit apoptosis induced by enforced p53 expression, can physically associate with Bax, implying that this oncoprotein interferes with p53-dependent apoptosis by antagonizing Bax function (Oltvai *et al.*, 1993; Moll *et al.*, 1996) As previous reported (Kurosawa *et al.*, 1997), Bcl-2 showed de novo expression in hepatocytes after BDL and the immunostaining intensity for Bcl-2 increased gradually. These results suggest that Bax function upregulated by p53 is inhibited by Bcl-2.

Fas is expressed in hepatocytes constitutively. Not all Fas-expressing cells, however, are susceptible to Fas-induced death signals, because sFas existing in the cytoplasmic compartment of the cells confers resistance to Fas-mediated apoptosis. Many cell lines express Fas predominantly in their cytoplasm and are hence resistant to anti-Fas-mediated apoptosis (Cheng *et al.*, 1994; Yano *et al.*, 1996). In spite of a growing body of evidence that only mFas can transduce apoptotic signals, limited data are available with regard to the cellular localization of Fas *in vivo*, especially in the bile salt-induced apoptosis model. Western blot analysis of Fas did not show any differences between controls and BDL rats in our experiments. Immunohistochemistry data shows that Fas is expressed predominantly in the cytoplasm following prolonged BDL. The activation of caspase 8, a downstream caspase to Fas receptor, did not occur in BDL rats. Furthermore, mRNA for mFas was expressed in control rats but decreased gradually and disappeared by ten days. The signals of the sFas mRNA was evident after ten days. These results indicate that Fas does not seem to play a role in apoptosis in this model, rather the increase in the soluble Fas in BDL rats may contribute to the adaptive protection of hepatocytes from the toxic bile salts.

In conclusion, our current studies provide *in vivo* evidence that apoptosis of hepatocytes during extrahepatic cholestasis in rats is independent of Fas and controlled by Bax expression. Considering the common features of apoptosis in BDL rat, our data may serve as an in vivo system for studies of molecular mechanisms of hepatoprotective drug in BDL rat model.

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