Arch Toxicol (2003) 77: 110–115
DOI 10.1007/s00204-002-0415-x

ORGAN TOXICITY AND MECHANISMS

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Changes in expression and immunolocalization of protein associated with toxic bile salts-induced apoptosis in rat hepatocytes

Received: 3 June 2002 / Accepted: 19 September 2002 / Published online: 22 November 2002

Abstract Cholestatic liver injury results from the accumulation of toxic bile salts within the liver. The aim of the present study was to examine the temporal changes in expression and immunolocalization of protein associated with apoptosis in cholestatic rat liver. Rats were anesthetized and cholestasis was induced by double ligation of the common bile duct and sectioning between the ligatures. The animals were euthanized at day 3 and at weeks 1, 2, 4, and 6 after bile duct ligation (BDL). Apoptotic cell death was increased fivefold after 3 days of BDL, decreased over 2 weeks, and remained constant thereafter as has been demonstrated by TUNEL staining. Western blot analysis for Bax, Bcl-2, cytochrome *c*, and p53 were performed. Results show that total cellular Bax protein was increased 3 days after BDL and decreased over time thereafter. We observed the translocation of Bax to mitochondria and subsequent release of cytochrome *c*. According to our immunohistochemical data, nuclear p53 increased 3 days after BDL, but cytoplasmic sequestration of p53 was observed after 1 week. The expression of c-Myc was inhibited by 3 days, but increased at later stages following BDL. Bcl-2 was increased over time in BDL rats. Our data suggest toxic bile salts-induced hepatocellular apoptosis is related to differential expression of Bcl-2 family member protein and release of cytochrome c. Cellular localization of p53 plays an important role in apoptotic death of hepatocytes in BDL rats.

Keywords Cholestasis · Apoptosis · Bax · Bcl-2 · Cytoplasmic sequestration of p53

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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 (Galle et al. 1995; Rodrigues et al. 1998, 1999; Faubion et al. 1999; Miyoshi et al. 1999).

Bcl-2 family proteins play a pivotal role in modulating cell homeostasis. Representatives of this family are Bcl-2 and Bcl-X_L, which inhibit apoptosis, and Bax, which stimulates cell death. In viable cells, a substantial portion of Bax is found in the cytosol or loosely attached to the membranes. Following a death signal, cytosolic Bax translocates to the mitochondria, resulting in the release of mitochondrial Cyt *c* into the cytosol (Narita et al. 1998; Gross et al. 1999). Cytochrome *c* release activates caspase 9 and subsequently activates caspase 3 in concert with the cytosolic factor Apaf-1 (Li et al. 1997). In primary rat hepatocytes, deoxycholic acid triggers Bax-mediated Cyt *c* release (Rodrigues et al. 1999). The same phenomenon was also demonstrated in an in vivo animal model fed with deoxycholic acid (Rodrigues et al. 1998). Miyoshi et al. (1999) reported that, in Fas-deficient lpr mice, hepatocyte apoptosis during extrahepatic cholestasis is associated with an increase in Bax expression and association of Bax with

mitochondria. Furthermore, the apoptosis of proliferating bile duct epithelial cells (BEC) induced by Roux-en-Y biliodigestive anastomosis after bile duct ligation (BDL), is related to de novo expression of Bax (Stähelin et al. 1999).

This study aimed to examine the expression and immunolocalization of apoptosis-related protein in toxic bile salts-induced hepatic injury. Our data suggest toxic bile salts-induced hepatocellular apoptosis is related to differential expression of Bcl-2 family member protein and release of Cyt *c*. Cellular localization of p53 plays an important role in apoptotic death of hepatocytes in BDL rats.

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 (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, and 6 after BDL. Sham-operated rats served as controls. Unoperated animals were used as day 0 controls. Studies using laboratory animals adhered to all of the applicable institutional and governmental guidelines for the humane care and use of laboratory animals.

Antibodies

Mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4) was purchased from Sigma Chemicals (Poole, UK). Mouse monoclonal antibodies against Bcl-2 and Bax were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Mouse monoclonal antibodies against p53 (clone PAb 240) and Cyt *c* were from Neomarkers (Fremont, Calif., USA) and Pharmingen (San Diego, Calif., USA), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG and mouse monoclonal anti-c-Myc were purchased from Zymed (San Francisco, Calif., USA). Horseradish peroxidase-conjugated goat anti-mouse IgG was a product of Boehringer Mannheim (Mannheim, Germany).

Measurement of serum biomarkers and histological examination

The levels of total bilirubin (TB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in serum were measured using an Autodry Chemistry Analyzer (Spotchem SP4410, Arkray, Japan). Fibrosis was confirmed by immunostaining of hepatic stellate cells and Western blot analysis of α-SMA protein.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

Apoptosis was detected by the TUNEL method using the Boehringer in situ death detection kit. 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 fluorescence microscope (magnification ×200).

Isolation of mitochondria

Livers were minced into small pieces on ice, resuspended in ice-cold homogenization buffer (70 mM sucrose, 220 mM mannitol, 1 mM EGTA, and 10 mM HEPES, pH 7.4), and gently homogenized. Mitochondria were separated by differential centrifugation and further purified by Sucrose-Percoll gradient centrifugation as described (Lieser et al. 1998).

Western blot analysis

Isolated liver tissues were homogenized in radioimmunoprecipitation (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 α-SMA, Bcl-2, Bax, c-Myc, and p53, tissue extracts (70 μg) were separated onto 12% acrylamide gels and then transferred on nitrocellulose membrane. The mitochondrial extracts for the detection of Bax and Cyt *c* were fractionated onto 15% acrylamide gels. Primary antibodies were diluted to 1:500 for α-SMA, Bax, Bcl-2, p53, Cyt *c*, and c-Myc and incubated with the blots. For mitochondrial Bax and Cyt *c*, 1:1,000 dilutions of the primary antibodies were used. The membranes were washed and incubated with secondary antibodies (1:1,500) for 1 h at room temperature. Proteins were visualized by electrochemiluminescence Western Blotting Detection System (Amersham, Aylesbury, UK).

Immunohistochemical analysis

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 1:100 dilution of anti-α-SMA and 1:50 dilution of anti-p53 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.

Results

Serum biochemistry and histopathology in BDL rats

In BDL rats, AST, ALT, and TB were increased after 3 days (Table 1). The levels of AST and ALT decreased after seven days, but TB remained unchanged until 14 days after BDL. Immunohistochemical staining of α-SMA demonstrated that ductular proliferation, and portal and periductular fibrosis increased progressively after BDL up to 6 weeks (Fig. 1A). The results were confirmed with Western blot analysis of α-SMA protein, in that the protein level was increased up to 6 weeks after BDL (Fig. 1B).

Induction of apoptosis

To analyze hepatocyte apoptosis in BDL rats, TUNEL staining was performed. There were no differences in the numbers of TUNEL-positive cells between control and sham-operated animals (data not shown). By 3 days after BDL, a fivefold increase in the number of hepatocytes with apoptosis was observed. The number of

Table 1 Serum biochemical values in rats with hepatic fibrosis induced by bile duct ligation (*BDL*). Serum biochemical values were measured by Autodry Chemistry Analyzer (Spotchem SP4410). Blood was obtained from control and BDL rats for the determination of alanine aminotransferase (*ALT*), aspartate aminotransferase (*AST*), and total bilirubin (*TB*). Data are mean ± SD

Group	Number	ALT (IU/l)	AST (IU/l)	TB (mg/100ml)
Normal control	4	49 ± 18	20 ± 8	0.2 ± 0.1
Sham control (3 days)	4	47 ± 7	13 ± 3	0.2 ± 0.1
Sham control (7 days)	4	64 ± 10	21 ± 4	0.3 ± 0.1
Sham control (14 days)	4	69 ± 14	28 ± 5	0.3 ± 0.1
BDL (3 days)	8	1,123 ± 168*, **	386 ± 76*, **	9.7 ± 1.1*, **
BDL (7 days)	6	428 ± 85*, ***, *****	109 ± 23*, ***, *****	7.8 ± 0.6*, ***, ******
BDL (14 days)	6	573 ± 76*, ****, *****	139 ± 24*, ****, *****	9.8 ± 1.4*, ****

*$P < 0.001$, significantly different from normal control group
**$P < 0.001$, significantly different from sham control (3 days) group
***$P < 0.001$, significantly different from sham control (7 days) group
****$P < 0.001$, significantly different from sham control (14 days) group
*****$P < 0.001$, significantly different from BDL (3 days) group
******$P < 0.05$, significantly different from BDL (3 days) group

TUNEL-positive cells decreased until 2 weeks and remained constant thereafter (Fig. 2).

Involvement of Bax in bile salts-induced hepatocellular apoptosis

We next analyzed the expression levels of Bcl-2 and Bax proteins. As expected from the report of Kurosawa et al. (1997), the expression of Bcl-2 increased with time after BDL, which seems a possible adaptive mechanism to resist bile salts-induced hepatocyte injury. Total cellular Bax protein was increased by 3 days after BDL and decreased over time thereafter (Fig. 3A).

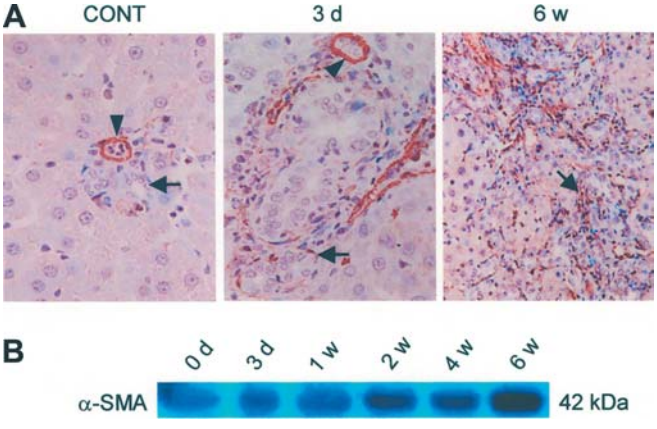

Fig. 1 **A** Immunohistochemical localization of α-smooth muscle actin (α-SMA) proteins. Liver tissues were obtained from bile duct-ligated (BDL) rats. Immunoreactivity was detected by the avidin-biotin complex method and 3-amino-9-ethylcarbazole, and counterstained with hematoxylin. *Arrowheads* and *arrows* indicate hepatic stellate cells around the blood vessels and proliferating bile ductules, respectively. **B** Western blot analysis of α-SMA proteins. Liver tissues were obtained from control and BDL rats. Seventy micrograms proteins were separated by 12% acrylamide gels, transferred to nitrocellulose membrane, and probed with mouse monoclonal anti-α-SMA antibody. This experiment was repeated three times and data from a representative experiment are shown

Cytosolic Bax translocates to the mitochondria following a death signal and then results in the release of mitochondrial Cyt *c* into the cytosol (Narita et al. 1998; Gross et al. 1999). To determine the translocation of Bax, we isolated mitochondria and analyzed for Bax and Cyt *c* expression. Bax was not detected in controls, but increased by 3 and 7 days after BDL. As Bax translocates to the mitochondria, the content of Cyt *c* decreased up to 7 days after BDL, but then recovered (Fig. 3B).

Localization of p53 and induction of apoptosis

It has been reported that Bax expression is regulated by p53 in many cell types (Miyashita and Reed 1995; McKenzie et al. 1999). In Western blot analysis, p53 protein levels increased by 3 days after BDL, and Bax

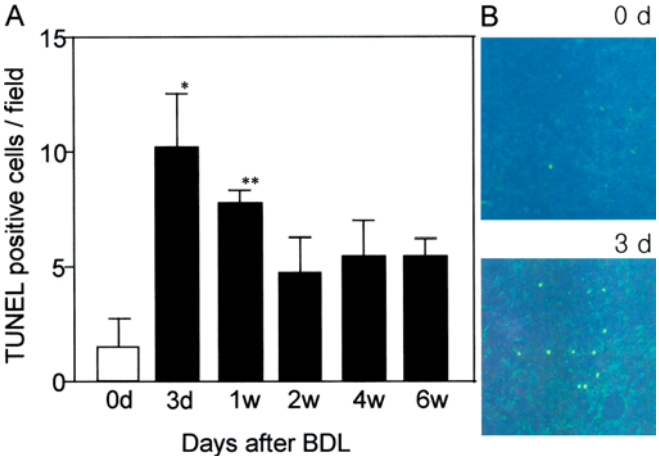

Fig. 2A, B Hepatocellular apoptosis in liver tissues of BDL rats. **A** Apoptotic cells were identified using the TUNEL method and visualized by fluorescence microscope (excitation: 490 nm; emission: 520 nm). The number of apoptotic cells was counted in 20 random microscopic fields. The values are means ± SEM. * $P < 0.02$, ** $P < 0.001$. **B** Representative TUNEL staining results from four to eight independent experiments are shown

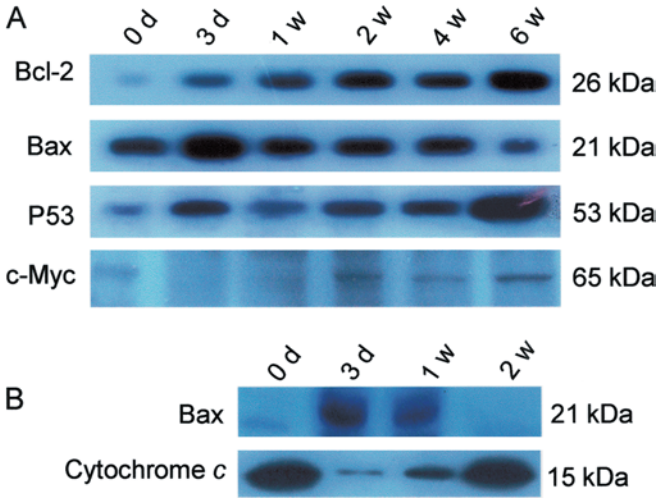

Fig. 3 Western blot analysis of total cellular Bcl-2, Bax, p53, and c-Myc proteins (**A**) and mitochondrial-associated Bax and cytochrome *c* (**B**). Liver tissues were obtained from control and BDL rats. Total cellular protein or mitochondrial protein was separated by 12–15% acrylamide gels, transferred to nitrocellulose membrane, and immunoblotted with the corresponding antibodies. Results are representative of three independent experiments

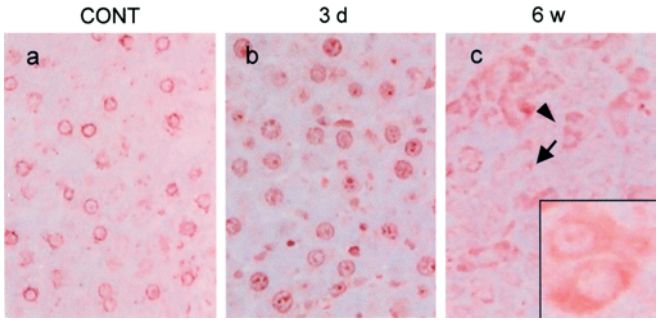

Fig. 4A–C Immunohistochemical localization of p53 protein. Liver tissues were obtained from control and BDL rats, and incubated with monoclonal anti-p53 antibody. Immunoreactivity was detected by avidin-biotin complex method and 3-amino-9-ethylcarbazole as a chromogen. p53 protein was expressed as three distinct patterns according to the time after BDL. In control rats, staining of p53 was observed in a discrete perinuclear ring (**A**). By 3 days, strong intranuclear staining was observed indicating that p53 is accumulated in the nucleus (**B**). By 6 weeks after BDL, p53 was expressed only in the cytoplasm of BEC (*arrow*) and hepatocytes (*arrowhead*) (**C**). *Inset* in **C** indicates a higher magnification of p53 staining. This experiment was repeated three times and data from a representative experiment are shown

expression was also increased at the same time. Unlike the expression kinetics of Bax, p53 protein showed enhanced expression at later stages following BDL (Fig. 3A). To explain this difference in the dynamic changes in the expression of Bax and p53, we performed immunohistochemical analysis of p53. Immunostaining of p53 appeared as conspicuous punctate structures in hepatocytes and BEC. For p53 to function as a transcription factor, it should be localized in the nucleus. In control rats, staining of p53 was observed in a discrete perinuclear ring (Fig. 4A). By 3 days after BDL, strong intranuclear staining was observed, indicating that p53 was accumulating in the nucleus (Fig. 4B). By 6 weeks, p53 was apparent in the cytoplasm of the hepatocytes with occasional nuclear staining, and thereafter it was expressed only in the cytoplasm of BEC and hepatocytes (Fig. 4C). Cytoplasmic staining of p53 was also observed at lower concentrations of antibody (1:100 and 1:200 dilution) indicating that the cytoplasmic sequestration of p53 protein was not an experimental artifact.

Reduced or inappropriate expression of c-Myc can be associated with cellular apoptosis. The expression of c-Myc was decreased by 3 days in BDL rats, and was increased gradually thereafter, which is in good accordance with the Bax expression profile.

Discussion

Biliary obstruction leads to inflammation and death of the hepatocytes. If cholestasis is prolonged, hepatic injury and cell death are progressive, with bile duct proliferation leading ultimately to liver fibrosis, cirrhosis, and cancer (Kountouras et al. 1984). It is now generally recognized that toxic bite salts induce apoptosis at the low concentrations that are typically observed during cholestasis. Recent investigations have focused on the elucidation of the cellular and molecular mechanisms of apoptosis by toxic bile salts. It is demonstrated that Fas receptors are involved in bile salt-induced hepatic cell death (Miyoshi et al. 1999; Sodeman et al. 2000).

Our data suggest that p53 was accumulated in the nucleus in acute biliary obstructed rats whereas cytoplasmic sequestration of p53 was observed in prolonged BDL rats. Nuclear p53 may regulate the expression of Bax in BDL rats as has been demonstrated in many cell types (Selvakumaran et al. 1994; Han et al. 1996; Xiang et al. 1998). The translocation of Bax to mitochondria supports the hypothesis that this may lead to release of Cyt *c* and transduce the signal for apoptotic death of hepatocytes by toxic bile salts.

Elevation of Bax protein level is induced in several clinically relevant settings where cell death occurs. 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 (Jurgensmeier et al. 1998; Narita et al. 1998). The data presented here suggest that cytoplasmic Bax translocates to the mitochondria at an early stage following BDL and induces release of Cyt *c* into the cytoplasm. The level of Bax in mitochondria was decreased by 7 days after BDL and the protein band was totally disappeared after 2 weeks. Similarly, the mitochondrial Cyt *c* content decreased immediately after BDL, increased gradually, and was restored to the control level by 2 weeks after BDL. The time course of the alteration of these two proteins in the mitochondria thus shows a good inverse correlation.

Wild-type p53 plays a pivotal role in cell growth, acts as a cell cycle checkpoint after DNA damage, and induces G1 arrest or apoptosis. The mechanism underlying its growth suppression is largely based on p53's function as a potent transcriptional regulator (El-Deiry et al. 1992; Funk et al. 1992). 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). In addition, 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). However, the role of p53 in the bile salts-mediated apoptosis in vivo has not been elucidated. There have been many reports on the cellular localization of wild-type p53 protein. This is a nuclear protein, and nuclear localization is essential for its growth suppressing activity. Cytoplasmic sequestration of wild-type p53 protein is observed in cases of mutagenesis, in mammalian models of viral oncogenesis, and some types of cancer. This type of p53 is functionally inactive as it fails to bind to its specific DNA target (Moll et al. 1996; McKenzie et al. 1999). In our immunohistochemical analysis using a p53-specific monoclonal antibody, reaction products were strictly localized to the perinuclear regions of the hepatocytes and BEC of the control rats. By 3 days after BDL, nuclear accumulation of p53 was observed. This increase in the expression of nuclear p53 may upregulate the production of Bax protein, which subsequently promotes apoptotic death of hepatocytes. The result is in agreement with the previous study by Bennett et al. (1998) in that human vascular smooth muscle cells undergo apoptosis in response to apoptotic activation via translocation of perinuclear p53 to the nucleus. One week after BDL, p53 protein began to be sequestered in the cytoplasm and this cytoplasmic staining was observed in most of the hepatocytes thereafter. Accordingly, the decrease in Bax expression in Western blot analysis and the decrease in the number of TUNEL-positive cells was observed at the same time point. Similarly, the reason that p53 failed to upregulate Bax expression at later stages of BDL can be found in our immunohistochemical data. Most p53 expression was observed in the cytoplasm, which lacks the transformation-suppressing and apoptosis-inducing activity of wild-type nuclear p53 (Shaulsky et al. 1991).

Evidence supports the idea that the reduced or inappropriate expression of c-Myc can be associated with cellular apoptosis (Bissonnette et al. 1992; Thompson 1998). Moreover, c-Myc and Bcl-2 can overcome p53-induced growth inhibition through exclusion of p53 from the nucleus during a critical period in the G1 phase of the cell cycle (Ryan et al. 1994). We have found that the expression of c-Myc was totally inhibited by 3 days after BDL but its expression was restored and increased gradually thereafter. There is good agreement in our results from the western blot analysis of Bax, Bcl-2, p53, and c-Myc proteins, immunohistochemical localization of p53, and the number of TUNEL-positive cells. Greim et al. (1972) found that the total amount of rat liver bile acids increases by fourfold at 3 days after BDL. The level of chenodeoxycholic acid, one of the major toxic bile acids responsible for the hepatocyte apoptosis, increases by threefold after 3 days and returns to almost normal at 8–10 days after BDL. The data correlate very well with the peak changes in the number of TUNEL-positive cells and the Bax as well as p53 expression seen at 3 days after BDL. Therefore, the results of our present study and of others (Ryan et al. 1994; Thompson 1998) strongly suggest that Bcl-2 and c-Myc inhibit hepatocellular apoptosis at later stages of BDL by sequestering p53 in the cytoplasm.

Our data suggest toxic bile salts-induced hepatocellular apoptosis is related to differential expression of Bcl-2 family member protein and release of Cyt *c*. Cellular localization of p53 plays an important role in apoptotic death of hepatocytes in BDL rats. Considering the common features of apoptosis in BDL rats, our data may serve as an in vivo system for studies of molecular mechanisms of hepatoprotective drugs in cholestatic liver diseases.

Acknowledgements This research was supported by a grant (PF002105–01) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Korean Government. The authors declare that the experiments comply with the current laws of Korea.

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