Salvia miltiorrhiza Inhibits Biliary Obstruction-Induced Hepatocyte Apoptosis by Cytoplasmic Sequestration of p53

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Cholestatic liver injury is caused by hepatocellular apoptosis induced by toxic bile salts. We have studied the effects of a traditional Chinese herbal medicine, Salvia miltiorrhiza, on apoptotic cell death in bile duct-ligated (BDL) rats. We also attempted to clarify the molecular mechanisms of the hepatoprotective effects of S. miltiorrhiza in this animal model. A water extract of S. miltiorrhiza (Sm-X; 200 mg/kg; po) was administered to BDL rats for 10 days. Rats were euthanized and apoptosis was detected in liver tissue by TUNEL staining. Western blot analysis and immunostaining for α-smooth muscle actin (α-SMA), Bax, Bcl-2, and p53 were performed. Results show that the treatment of BDL rats with Sm-X significantly improved the liver function parameters, although the expression of α-SMA, a marker of hepatic stellate cell activation, was not affected. Treatment with Sm-X significantly reduced the number of apoptotic cells. A time-dependent decrease in Bax protein level and an increase in Bcl-2 protein level were observed in BDL rats treated with Sm-X. Immunohistochemical analysis demonstrated that p53 was strongly positive in hepatocyte nuclei of BDL rats but that treatment with Sm-X induced a cytoplasmic sequestration of p53. Taken together, hepatoprotective effects of Sm-X partially ascribe to the antiapoptotic property in hepatocytes. Treatment of Sm-X-induced cytoplasmic sequestration of p53, downregulation of Bax, and upregulation of Bcl-2 protein. This study identifies and delineates signaling factors involved in the antiapoptotic properties of Sm-X and suggests a potential application of S. miltiorrhiza in the clinical management of hepatic disease induced by toxic bile salts following biliary obstruction. © 2002 Elsevier Science (USA)

Key Words: Salvia miltiorrhiza; bile duct ligation; apoptosis; Bax; Bcl-2; p53; cytoplasmic sequestration.

Salvia miltiorrhiza BUNGE is a traditional Chinese herbal medicine used for centuries to improve blood circulation. S. miltiorrhiza has been widely used in oriental medicine for the treatment of chronic hepatitis and liver fibrosis. In rats, components of a water-soluble extract of S. miltiorrhiza prevent peroxidative damage to liver microsomes, hepatocytes, and erythrocytes (Liu et al., 1992). Lithospermate B, one of the active ingredients of S. miltiorrhiza, exerts a protective effect against cell death in experimental hepatitis induced by carbon tetrachloride and D-galactosamine/lipopolysaccharide (Hase et al., 1997). Tanshinone I and tanshinone II-A, other components isolated from the root of S. miltiorrhiza, are effective antioxidants that inhibit lipid peroxidation (Cao et al., 1996; Ng et al., 2000). It has also been reported that, by inducing apoptosis of tumor cells and by preventing aflatoxin B1-induced hepatocarcinogenesis in rats, S. miltiorrhiza possesses chemopreventative potential (Liu et al., 2000, 2001).

The failure of bile salt excretion in cholestasis leads to retention of hydrophobic bile salts within the hepatocytes (Greim et al., 1972a,b) and causes apoptosis and/or necrosis (Myoshi et al., 1999). Although the mechanism of bile salt-mediated apoptosis is not completely understood, the involvement of the Fas death receptor has been suggested by in vitro studies, as well as by in vivo studies using an animal model with bile duct ligation (BDL) (Myoshi et al., 1999; Faubion et al., 1999).

Following a death signal, mitochondrial cytochrome c is released into the cytoplasm (Gross et al., 1999; Narita et al., 1998), which activates caspase 9, and this subsequently activates caspase 3 in concert with the cytosolic factor Apaf-1 (Li et al., 1997). In Fas-deficient lpr mice, hepatocyte apoptosis during extrahepatic cholestasis is associated with an increase in Bax expression and association of Bax with mitochondria (Myoshi et al., 1999). The apoptosis of proliferating bile duct epithelial cells (BEC) induced by the Roux-en-Y biliodigestive anastomosis after BDL is related to de novo expression of Bax (Stähelin et al., 1999).

Wild-type p53 is a tumor suppressor protein, which functions as a transcription factor downregulating cell proliferation and inducing apoptosis in response to various types of cellular stress (Levine, 1997; Ko and Prives, 1996). It has been suggested that one of the mechanisms of inhibition of apoptosis is related to the cytoplasmic sequestration of p53 (Kovacs et al., 1996). This type of p53 is functionally inactive, as it fails to

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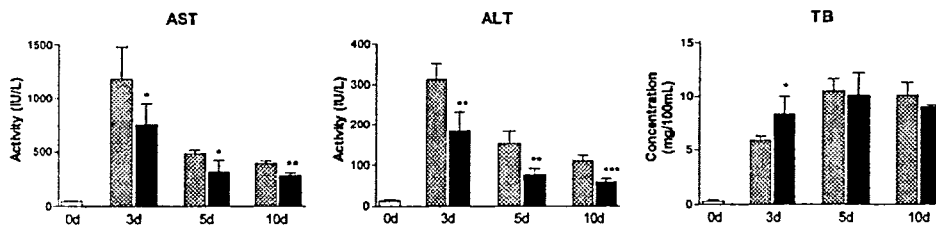[2] To whom correspondence should be addressed.

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FIG. 1. Serum biochemical parameters in Sm-X-treated BDL rats. Blood was obtained from sham control (□), BDL control (▨), and Sm-X-treated BDL rats (■) for the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB) using an Autodry Chemistry Analyzer. Data are means ± SD of five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from BDL control rats.

bind to its specific DNA target. Thus, nuclear localization of p53 after translation in the cytoplasm is essential for its normal activity (Shaulsky et al., 1991). We have reported previously that a hot water extract of S. miltiorrhiza (Sm-X) inhibits fibrosis and lipid peroxidation in BDL rats (Nan et al., 2001). In the present study, we attempted to clarify the molecular mechanisms underlying the hepatoprotective effects of Sm-X in BDL rats. Of particular interest to this investigation was the expression and cellular localization of p53 and Bcl-2 family proteins in terms of the regulation of hepatocellular apoptosis in vivo.

MATERIALS AND METHODS

Preparation of Sm-X. The roots of S. miltiorrhiza were purchased from Sungboe Farm Co. (Ahnsung, Kyunggi-do, Korea) and authenticated by Professor Y.C. Kim, College of Pharmacy, Wonkwang University. Sm-X was prepared by boiling the dried roots with distilled water for 5 h. The extract was filtered, freeze-dried, and kept at 4°C. The extraction yield was approximately 8.1% (w/w). The dried extract was dissolved in distilled water before use.

Biliary obstruction and animal treatments. Specific pathogen-free male Sprague-Dawley rats (200–220 g) were obtained from Daehan Laboratory Animal Research 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. After animals were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), bile duct ligation was performed by double ligation of the common bile duct and sectioning between the ligatures. After surgery, Sm-X was dissolved in distilled water and administered daily (200 mg/kg po) over the experimental period. Control rats received equal volumes of the solvent and sham-operated animals were used as day 0 controls. Animals were euthanized at 3, 5, and 10 days after BDL.

Antibodies. Mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4) was purchased from Sigma Chemical Co. (Poole, UK) and mouse monoclonal antibodies against Bcl-2 and Bax were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against p53 (clone PAb 240) and anti-mouse IgG were obtained from Neomarkers (Fremont, CA) and Zymed (San Francisco, CA), respectively.

Measurement of serum biomarkers. Blood was obtained by cardiac puncture for serum biochemical analysis. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as the content of total bilirubin (TB) in the serum, were measured using an Autodry Chemistry Analyzer (Spotchem SP4410, Arkray, Japan).

In situ nick-end labeling for DNA fragmentation. Apoptotic cells were detected by the method of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL, fluorescein), using a commercial in situ death-detection kit (Boehringer, Manheim, Germany). Formalin-fixed liver tissues were dehydrated through increasing concentrations of ethanol and then embedded in paraffin. Sections (4 μm) were deparaffinized in xylene. After sections were rehydrated in phosphate-buffered saline, they were incubated with proteinase K (20 μg/ml in Tris–HCl) for 10 min at room temperature and processed according to the manufacturer's protocol. The number of TUNEL-positive cells was counted in 20 random microscopic fields (200×) for each specimen using a confocal microscope (Fluoview, Olympus). Indirect TUNEL labeling assay was also performed using a TACS2 TDT-DAB in situ apoptosis detection kit (Trevigen, Gaithersburg, TN) to identify the cell types undergoing apoptosis. Color development was performed with 3–3'-diaminobenzidine-tetrahydrochloride and the sections were counterstained with methyl green.

Immunoblots for α-SMA, Bax, Bcl-2, and p53. Isolated liver tissues were homogenized in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM PMSF, and 5 mM EDTA). After the homogenates were centrifuged at 4°C (600g for 15 min), the supernatants were collected and the protein concentration was measured. Aliquots of cytosolic protein from each sample were separated onto a 12% acrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with mouse monoclonal anti-α-SMA, anti-Bax, anti-Bcl-2, and anti-p53 (1:500 dilution) for 1 h. The membrane was incubated with secondary antibodies (1:1500 dilution) for 1 h and the blots were visualized by electrochemiluminescence using the Western Blotting Detection System (Amersham, UK).

Immunohistochemical analysis of α-SMA, Bax, Bcl-2, and p53. Section preparation was initially the same as described for TUNEL assay. The sections were then subjected to antigen retrieval by microwave (700 W for 2 min) in citrate buffer (pH 6.0). Proteins were detected by avidin–biotin complex staining. Primary antibodies for α-SMA (1:300), Bax (1:50), Bcl-2 (1:50), and p53 (1:50) were applied to sections overnight at 4°C. Alternate sections were treated with normal rabbit serum as a control. Sections were further treated with secondary antibody. Peroxidase activity was detected with 3-amino-9-ethylcarbazole before counterstaining sections with hematoxylin.

RESULTS

Effect of Sm-X on Liver Function Parameters in Serum

To test the general liver function of sham control, BDL control, and Sm-X- treated BDL rats, serum levels of AST, ALT, and TB were determined utilizing specific enzyme assays. As shown in Fig. 1, serum AST and ALT were increased

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at 3 days in BDL rats; the levels decreased thereafter. The TB level was increased and remained constant until 10 days after BDL. Treatment with Sm-X lowered the activities of AST and ALT significantly. The TB level was slightly increased by treatment with Sm-X at day 3, but remained unchanged thereafter.

Expression of α-SMA

Nonparenchymal hepatic stellate cells (HSC) are activated in response to liver damage. They produce type I collagen leading to hepatic fibrosis and also express the intracellular microfilament protein α-SMA. Therefore, the effect of Sm-X on the expression of α-SMA in BDL rats was determined by Western blot analysis and immunostaining of HSC. In BDL rats, expression of α-SMA increased with time. However, treatment with Sm-X for 10 days did not affect the activation of HSC (Fig. 2).

Prevention of Apoptotic Cell Death by Sm-X

TUNEL staining was performed to analyze hepatocellular apoptosis using the direct fluorescein-labeling cell death detection kit. As shown in Fig. 3A–C, a five- to sevenfold increase in the number of hepatocytes undergoing apoptosis was observed at 3 and 5 days after BDL. Treatment with Sm-X significantly decreased the number of apoptotic cells in BDL rats. To identify the cell types undergoing apoptosis in BDL rats, we performed indirect TUNEL assay using the TACS2 TDT-DAB *in situ* apoptosis detection kit. We observed that only hepatocytes, and not HSC or BEC, were undergoing apoptosis in BDL rats (Fig. 3D). The number of apoptotic hepatocytes was reduced significantly by treatment with Sm-X (Fig. 3E).

Expression of Bax and Bcl-2 Proteins by Treatment with Sm-X

The expression of proapoptotic Bax and antiapoptotic Bcl-2 was monitored in BDL control and Sm-X-treated BDL rats. Following BDL, total cellular Bax protein initially increased at 3 and 5 days and then decreased at 10 days (Fig. 4A). Treatment of BDL rats with Sm-X further decreased the expression of Bax to the control level. Parallel to the results of Western blot analysis, the staining intensity of Bax increased in BDL rats and then decreased dramatically after treatment with Sm-X (Fig. 5A–C). Antiapoptotic Bcl-2 protein is known to be expressed in BDL rats as an adaptive mechanism to resist hepatocyte damage induced by toxic bile salts. As was anticipated, the level of Bcl-2 protein increased over time in BDL rats. This increase in Bcl-2 expression then became more pronounced in BDL rats treated with Sm-X (Fig. 4A). The increased ratio of Bax/Bcl-2 in BDL rats was reduced by treatment with Sm-X in a time-dependent manner (Fig. 4B). In BDL rats, most Bcl-2 was expressed in BEC surrounding bile ductules (Fig. 5E).

However, treatment with Sm-X increased Bcl-2 expression in hepatocytes rather than in BEC (Fig. 5F).

Cytoplasmic Sequestration of p53 by Sm-X

To test the relationship between Bax and p53 protein and to compare the time course of the apoptotic protein expression, we performed Western blot and immunohistochemical analysis of p53. It has been reported that Bax expression is regulated by p53 in many cell types. Consistent with upregulation of Bax expression in BDL rats, an increase in p53 protein was observed by Western blot analysis (Fig. 4A). In Sm-X-treated rats, however, the expression of p53 was not reduced in the Western blot analysis. This discrepancy between Bax and p53 protein expression in Sm-X-treated rats could be explained by the immunohistochemical analysis. At 3 and 5 days after BDL, strong intranuclear staining was observed, indicating that p53 had accumulated in the nucleus (Figs. 5H and 5I). By 10 days, when the apoptotic index had decreased to 50% of maximum, there was some sequestering of p53 in the cytoplasm and in the perinuclear region (data not shown). However, most p53 still remained in the nucleus.

In the Sm-X-treated BDL rats, we could observe that all of the p53 was sequestered in the cytoplasm (Fig. 5J). From these results, we can conclude that treatment of BDL rats with Sm-X protects hepatocytes from apoptosis by downregulating Bax and upregulating Bcl-2 expression. The time course of Bax expression correlates well with the relocation of p53 from the nucleus to the cytoplasm.

DISCUSSION

The aim of the present investigation was to demonstrate the effect and to determine the possible molecular mechanisms of Sm-X on hepatocyte injury induced by bile salts in BDL rats. It has been demonstrated that the BDL rat model shows almost no generation of toxic intermediates, no massive hepatocyte necrosis, and no significant inflammation (Muriel *et al.*, 1994). BDL induces a type of liver damage that is etiologically and pathogenetically different from the experimental liver damage induced by poisons such as carbon tetrachloride (Kountouras *et al.*, 1984). The BDL model therefore allows the detection of antiapoptotic drug effects. Using the BDL model or toxic bile salts in primary hepatocyte culture, several researchers have examined the antifibrotic and hepatoprotective effects of apoptosis-modulating agents. Interferon-α and tetrandrine prevent hepatic fibrosis in BDL rats (Bueno *et al.*, 2000; Park *et al.*, 2000). Hydrophilic bile acids, such as ursodeoxycholic acid and tauroursodeoxycholic acid, reduce the incidence of apoptosis in hepatocytes induced by toxic bile salts (Benz *et al.*, 1998, 2000); antioxidants exert the same effect (Yerushalmi *et al.*, 2001).

The results of our present study demonstrate that the treatment of BDL rats with Sm-X prevents hepatocellular apoptosis
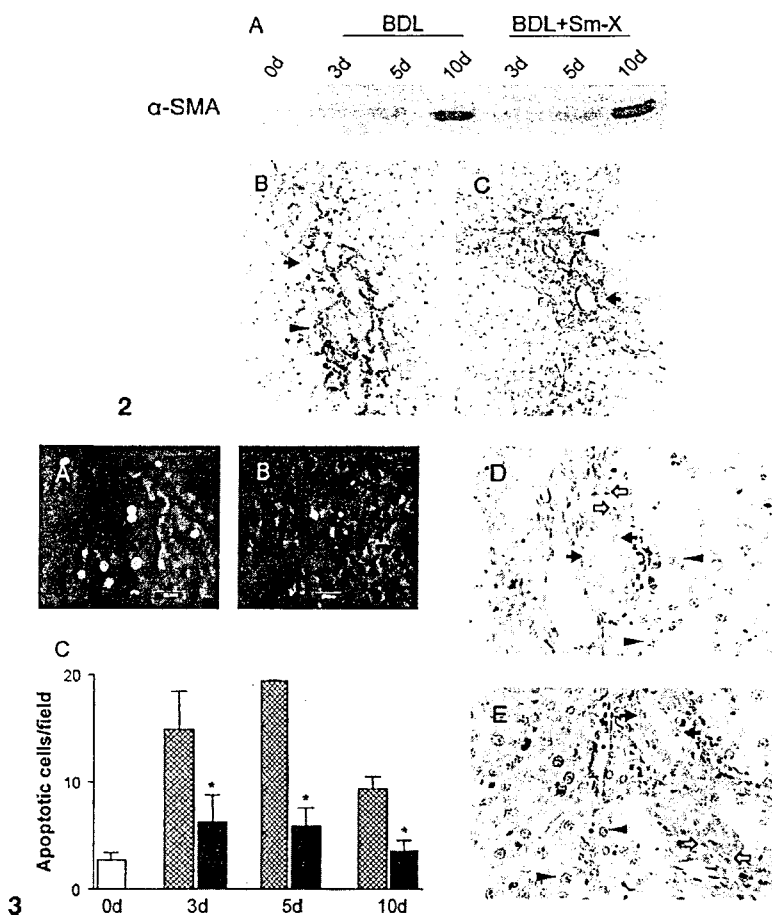
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FIG. 2. Western blot and immunohistochemical analysis of α-smooth muscle actin (α-SMA) protein. Liver tissues were obtained from sham control, BDL control, and Sm-X-treated BDL rats. Seventy-microgram proteins were separated onto a 12% acrylamide gel, transferred to a nitrocellulose membrane, and probed with mouse monoclonal anti-α-SMA antibody (A). For immunohistochemical analysis, immunoreactivity was detected by avidin–biotin complex. Primary and secondary antibodies were applied to sections and peroxidase activity was detected with 3-amino-9-ethylcarbazole before counterstaining sections with hematoxylin. Activated stellate cells are seen around the blood vessels (➡) and proliferating bile ductules (▶) of BDL control (B) and Sm-X-treated BDL rats (C). Data are representative of five independent experiments.

FIG. 3. Antiapoptotic effects of Sm-X in BDL rats. Apoptotic cells were detected using the direct fluorescein-labeling cell death detection kit (A, BDL control; B, BDL + Sm-X), and the number of apoptotic cells was counted in 20 random microscopic fields of sham control (□), BDL control (▨), and Sm-X-treated BDL rats (■). The values are means ± SD of five independent experiments (*$p < 0.001$) (C). The cell types undergoing apoptosis were identified using a TACS2 TdT-DAB *in situ* apoptosis detection kit (D, BDL control; E, BDL + Sm-X). Only hepatocytes (▶) and not hepatic stellate cells (▷) or bile duct epithelial cells (➡) were undergoing apoptosis in BDL rats. TUNEL stainings are representative of at least five independent experiments.

in vivo. Although the hepatoprotective and antifibrotic effects of *S. miltiorrhiza* or its constituents have been demonstrated in various cell types and animal models (Nan *et al.*, 2001; Cao *et al.*, 1996; Wang, 2000), the molecular mechanisms by which *S. miltiorrhiza* protects the hepatocyte from toxic bile salt-induced damage are largely unknown.

According to our previous results, the treatment of BDL rats with Sm-X for 4 weeks inhibits hepatic fibrosis and lipid peroxidation (Nan *et al.*, 2001). Sm-X contains a mixture of natural phenolic compounds, which have antioxidant effects *in vitro* and *in vivo* (Cao *et al.*, 1996; Lin *et al.*, 1996; Zhou *et al.*, 1999). It is widely accepted that the severity of hepatic damage

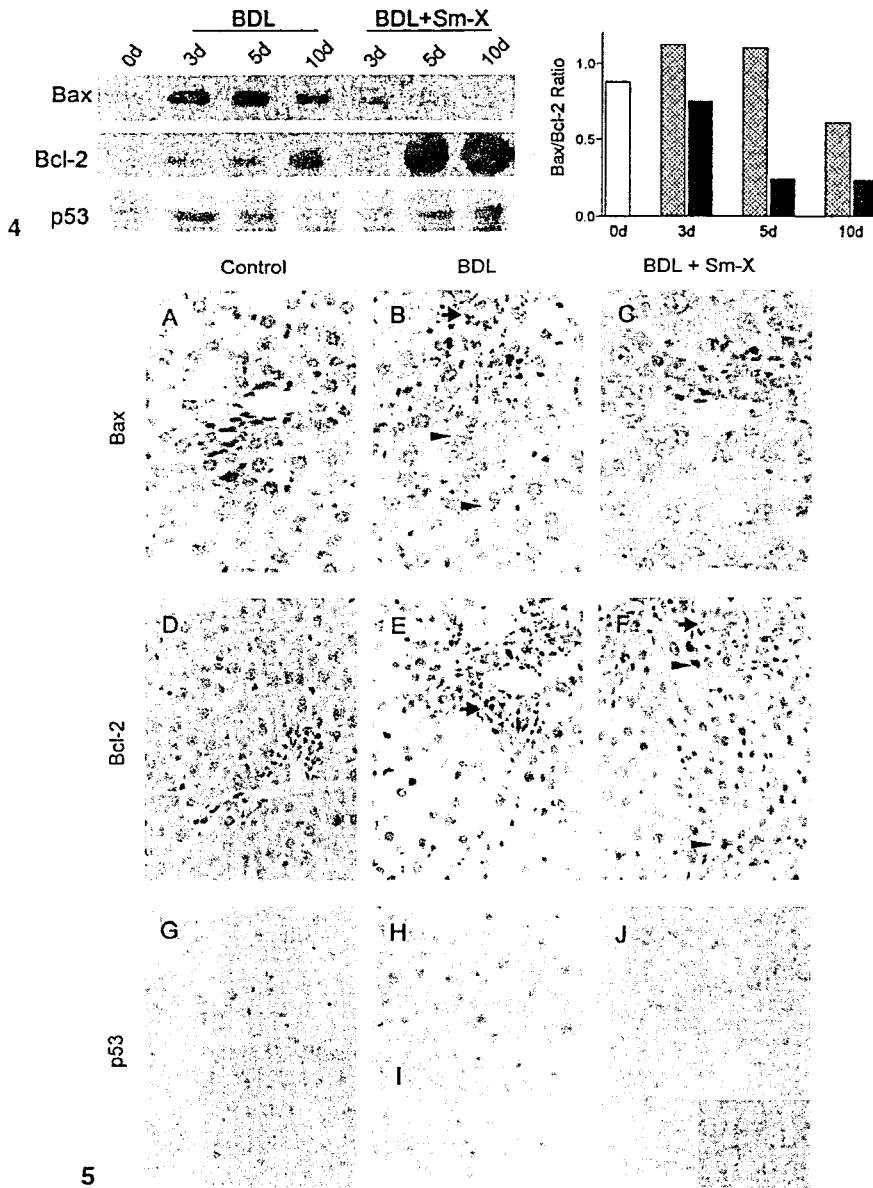ANTIAPOPTOTIC PROPERTY OF *S. Miltoirrhiza* IN BILIARY OBSTRUCTION

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FIG. 4. Western blot analysis of Bax, Bcl-2, and p53 proteins. Liver tissues were obtained from sham control, BDL control, and Sm-X-treated BDL rats. Total cellular protein was separated onto a 12% acrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with the corresponding antibodies. The blots were visualized by the electrochemiluminescence Western Blotting Detection System (A). The Bax/Bcl-2 ratio was obtained by densitometric analysis of each blot from sham control (☐), BDL control (▨), and Sm-X-treated BDL rats (■) (B). Results are representative of at least five independent experiments.

FIG. 5. Immunohistochemical localization of Bax, Bcl-2, and p53 protein. Liver tissues were obtained from sham control (A, D, and G), BDL control (B, E, H, and I), and Sm-X-treated BDL rats (C, F, and J) and incubated with corresponding antibodies. Bax expression increased in hepatocytes (►) and bile duct epithelial cells (➡) of BDL liver tissues obtained at 5 days (B) but decreased dramatically after treatment with Sm-X (C). In BDL rats, Bcl-2 was expressed mainly in bile duct epithelial cells (➡) at 5 days (E). The treatment of Sm-X increased the Bcl-2 expression in hepatocytes (►) (F). p53 protein had accumulated in the nucleus in BDL control rats at 3 (H) and 5 days (I). Treatment of Sm-X to BDL rats induced cytoplasmic sequestration of p53 (J). (Inset: Higher magnification of the hepatocytes showing cytoplasmic sequestration of p53). Data are representative of at least five independent experiments.

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in individuals or in experimental animals with biliary obstruc-
tion is causally associated with the extent of intrahepatic oxi-
dative stress (Ljubuncic *et al.*, 2000; Baron and Muriel, 1999).
Increased levels or accelerated generation of reactive oxygen
species and toxic degradative products of lipid peroxidation
induce hepatic cell death. Based on these findings, we have
postulated that the antioxidative properties of Sm-X may con-
tribute to the protection of the liver from damage induced by
toxic bile salts in BDL rats.

Contrary to the results obtained in the 4-week-treatment
study, short-term treatment of BDL rats with Sm-X for 10 days
did not affect the expression of α-SMA, a marker of hepatic
stellate cell activation during the fibrogenic process. We have
therefore studied the acute protective effects of Sm-X on
hepatocyte apoptosis in BDL rats. Treatment with Sm-X for at
least 3 days decreased the number of TUNEL-positive cells
dramatically. After 10 days of treatment, the number of apo-
ptotic cells had decreased to almost the control level.

Elevation of the Bax protein level is induced in many
clinically relevant settings where cell death occurs. Bcl-2 is an
apoptosis suppressing factor that heterodimerizes with Bax and
neutralizes the protective effects of the latter. When Bcl-2 is
present in excess, cells are protected from apoptosis. In con-
trast, when Bax is in excess and the homodimers of Bax
dominate, cells are susceptible to programmed cell death.
Therefore, it is the ratio of Bax to Bcl-2 that determines the fate
of a cell, rather than the absolute concentrations of either
(Gross *et al.*, 1999). In BDL rats, the Bax/Bcl-2 ratio is
elevated at 3 and 5 days after BDL, but decreased at 10 days.
The time course of changes in the Bax/Bcl-2 ratio correlates
very well with the time course of the number of TUNEL-
positive cells. According to Greim *et al.* (1972a), the total
amount of rat liver bile acids is increased fourfold 3 days after
BDL. The level of chenodeoxycholic acid, one of the major
toxic bile acids responsible for hepatocyte apoptosis, increases
threefold at 3 days after BDL and returns to almost normal at
8–10 days after BDL. Thus, the kinetic change in the concen-
tration of bile acids is in good accord with our present data.
Treatment with Sm-X reduced the expression of Bax and
increased the expression of Bcl-2 significantly, thereby reduc-
ing the Bax/Bcl-2 ratio. Moreover, the expression of Bcl-2 was
not restricted to BEC. Under treatment with Sm-X, Bcl-2 was
also expressed in hepatocytes. This result indicates that, as well
as BEC, with which bile salts are mainly in contact, hepato-
cytes are also protected from apoptosis by treatment with
Sm-X.

Wild-type p53 protein plays a key suppressor role in cell
growth and tumor formation and acts as a cell cycle checkpoint
after DNA damage induces G1 arrest or apoptosis (Levine,
1997; Ko and Prives, 1996). One of the transcriptional targets
of p53 that may be important for apoptosis is Bax. Overex-
pression 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). Wild-type p53 is a
nuclear protein and nuclear localization is essential for its
growth-suppressing activity. Cytoplasmic sequestration of
wild-type p53 protein has been reported in mammalian models
of viral oncogenesis and atherogenesis (Wang *et al.*, 2001) and
in certain types of cancer (Moll *et al.*, 1995). Cytoplasmic p53
is functionally inactive because it fails to bind to its specific
DNA target. Thus, cytoplasmic p53 protect cells from death, as
has been shown in many tumors (Moll *et al.*, 1996; McKenzie
et al., 1999). In mammary epithelium and in a neuroblastoma
cell line, treatment with retinoic acid or hormones reverses the
cytoplasmic sequestration and functional repression of p53
(Kuperwasser *et al.*, 2000; Takada *et al.*, 2001), thereby sug-
gesting that the functional status of p53 can be altered and
regulated by specific stimuli. In our immunohistochemical
analysis based on a p53-specific monoclonal antibody, reaction
products in rats receiving Sm-X were observed only in the
cytoplasm. Consistent with the cytoplasmic sequestration of
p53, the expression of Bax decreased following treatment with
Sm-X.

In cases of extrahepatic cholestasis, the use of *S. miltiorrhiza*
may provide an effective prophylactic and therapeutic tool by
inhibiting apoptosis in hepatocytes. The elucidation of the
properties of the factors involved in the interactions of p53 in
hepatocytes may uncover the mechanism of action of the
antiapoptotic effects of Sm-X. The results presented in this
report may open the window to develop a new therapeutic
strategy in cholestatic liver injury.

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