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Alteration of X-linked Inhibitors of Apoptosis (XIAP) Expression in Rat Model with DEN-induced Hepatocellular Carcinogenesis

Jae-Jin Chang[1,2], Su-Yeon Jeon[3], Ji-Ye Song[3], Jin-Hee Kim[3] , Lan Li[2], Dae-Hun Park[4], Yun-Lyul Lee[5], Jeong-Joo Park[2], Dong-Wook Woo[2], Gi Jin Kim[3] & Min-Jae Lee[2]

[1]Orient Bio Inc. Seongnam, Gyeonggi-Do, Korea
[2]College of Veterinary Medicine, Kangwon National University, Chuncheon, Korea
[3]Graduate School of Life Science and Biotechonology, College of Medicine, Pochon CHA University, Seoul 135-081, Korea
[4]Development of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Daejeon, Korea
[5]Department of Physilolgy, College of Medicine, Hallym University, Chuncheon, Korea
Correspondence and requests for materials should be addressed to M. J. Lee (mjlee@kangwon.ac.kr)

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Abstract

The X-linked inhibitor of apoptosis (XIAP) is a member of a novel family of inhibitors of apoptosis and has several BIR domains (BIR1, BIR2, and BIR3) and a carboxy-terminal RING zinc-finger. Since suppression of apoptosis is fundamentally important for carcinogenesis and tumor growth, we investigated the expression and function of XIAP in DEN-induced carcinogenesis using rat model. Wistar rats were injected intraperitoneally with DEN at a dose of 50 mg/kg in twice a week for 12 weeks (Group II) and 16 weeks (Group III) followed by the recovery periods, respectively. The evaluation of DEN-induced carcinogenesis carried out the blood, RT-PCR, histopathological and western blot analysis. The level of blood chemistry including GOT/GPT, albumin, and total bilirubin were significantly exchanged comparing to control and Group I/Group II. The expression of albumin and collagen mRNA were significantly exchanged ($P<0.05$) in both groups. In addition, AFP mRNA expression decreased more after recovery periods than Group II. XIAP was expressed constitutively in normal rat liver as well as DEN-induced Groups I and Group II. In addition, XIAP expression increased more in Group I with 4 weeks recovery periods than Group I. However, XIAP expression shown to increase in Group II, otherwise, it was decreased in Group II with 10 weeks repair periods. Taken together, these results suggest the alteration of XIAP expression could be involved in hepatocellular carcinogenesis.

Keywords: X-linked Inhibitors of Apoptosis (XIAP), N,N-Diethylnitrosamine (DEN), Carcinogenesis, Apoptosis

Hepatocellular carcinoma (HCC), a major type of primary liver cancer, is one of the most frequent human malignant neoplasmas[1,2]. Common risk factors of human HCC include chronic hepatitis B/C virus infection, dietary aflatoxin B1 (AFB1) ingestion, chronic alcohol abuse, and cirrhosis associated with liver diseases as well as a close correlation between epigenetic regulation and environmental factors[3,4]. In human hepatocarcinogenesis, imbalance between cell proliferation and death pathways lead to a protumorigenesis[5,6].

Apoptosis is an essential biologic process to several physiologic and pathologic conditions and contribute to the appearance of a hepatic disease through the mechanism of development and homeostasis in response to stimuli that is abnormal[7-10]. Among the genes controlling apoptosis, inhibitor of apoptosis proteins (IAP) was identified from baculoviruses[11], and neuronal apoptosis inhibitory protein (Naip), X-linked inhibitor of apoptosis protein-1 (XIAP), human inhibitor of apoptosis protein-1 (Hiap-1), Hiap-2, and surviving, belongs to a family of IAP, which have been in mammalian cells[12,13]. Especially, the X-linked inhibitor of apoptosis, XIAP, is egulated by XIAP-associated factor-1 (XAF1) followed act as caspase inhibitory mechanisms[14,15]. Low XAF1 and XAIP expressions are correlated to poorly differentiated HCC[16-18]. Otherwise, Shiraki K *et al*. have reported that abundant XIAP protein expressed in a number of human cancers, including hepatocellular carcinoma[19,20]. Thus, the regu-

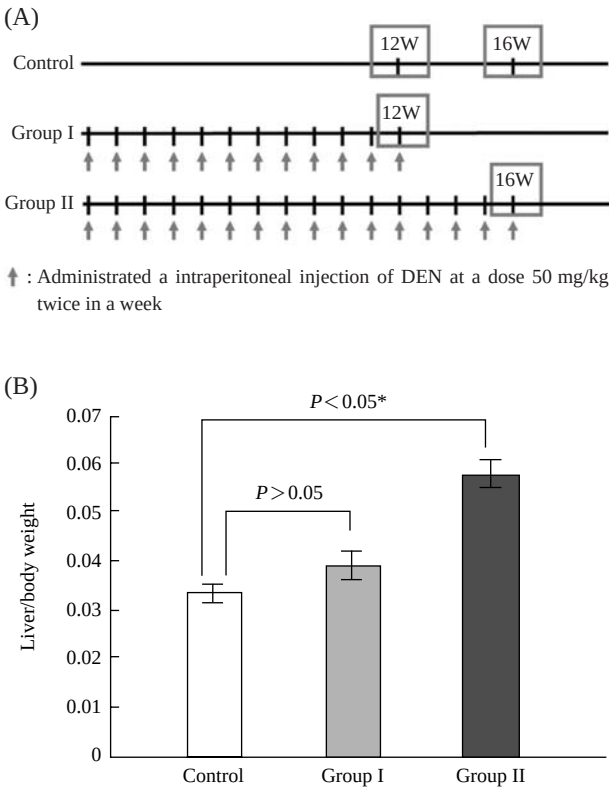

Figure 1. Generation of DEN-induced hepatocellularcarcinogenesis model. (A) The schedules of DEN administration are to generate DEN-induced HCC model. Arrow means administration into intraperitoneal injection of DEN at a dose 50 mg/kg twice in a week. (B) The ratio of liver/body weight in control group and DEN-induced HCC groups. Data are expressed at the mean ± SD. Statistical analysis was carried out by unpaired student's t-test (*$P < 0.05$).

lation and the function of XIAP expression in HCC are not clear.

Due to the reason, many researchers have been generated several types of hepatocellular carcinoma in animal models using various carcinogens or chemicals such as dimethylnitrosamine (DMN), carbon tetrachloride (CCl_4) and thioacetamide (TAA) for the correlation between hepatocellular carcinogenesis and XIAP expression. Unfortunately, it is difficult to evaluate hepatocellular carcinogenesis in through chemical-induced animal model because the several variations for animal condition and different mechanisms of chemical-induced carcinogenesis. Therefore, the evaluation of hepatocellular carcinoma animal model is critical point to explore the specific gene expression and function in HCC including get the consistent results. In addition, expressions of XIAP in animal HCC model have not been reported *in vivo*.

This study was conducted to determine whether XIAP mRNA and protein expressions are altered in the DEN-induced hepatocellular carcinoma rat model. The alteration of XAIP expression in DEN-induced HCC was also analyzed to assess possible link of the evaluation of DEN-induced HCC rat model.

Generation of DEN-induced Hepatocellular Carcinoma in Rats

To induce hepatocellular carcinogenesis in rats, DEN at 50 mg/kg of body weight was injected into the intraperitoneally twice a week for 12 weeks (Group I) and 16 weeks (Group II) followed by the recovery periods. Grouping and a scheme of the experiments are shown in Figure 1. The liver/body weight ratio in rats given DEN for 16 weeks were significantly increased ($P < 0.05$) than rats with DEN for 12 weeks ($P > 0.05$) and Control (Figure 2A). We also observed massive appearances of the liver in rats with DEN-treated for 16 weeks than Control and DEN-treated liver for 12 weeks (data not shown).

Sequential Development of Cirrhosis and HCC in Rats Model with DEN-induced Liver Injury

For analysis the changes for the function and the structure of liver, RT-PCR was carried out with primers for hepatocyte-specific markers and α-SMA in Control and DEN-treated groups (Figure 2B). The expression of AFP mRNA was increased in DEN-induced groups. The alterations of AFP mRNA expression were observed in DEN-induced groups with recovery periods than those without recovery periods. Especially, decreased AFP mRNA expression on DEN-treated liver for 16 weeks with repair was observed ($P < 0.5$). We examined for α-SMA mRNA expression in liver samples to confirm the liver injury through collagen deposition in liver tissue. The expressions of α-SMA were progressed in DEN-treated group for 16 weeks than 12 weeks, otherwise, those with recovery periods were increased in DEN-treated group for 12 weeks than 16 weeks. However, the expressions of albumin mRNA were significantly decreased in DEN-treated groups than control regardless of the recovery periods ($P < 0.5$).

We then examined whether the alteration of XIAP mRNA expression in DEN-treated group were detected by RT-PCR (Figure 3). There was only little expression of XIAP mRNA shown in control group. However, the XIAP mRNA expression increased in DEN-treated group for 12 weeks and 16 weeks recovery periods. Especially, the expression of XIAP was significantly increased in DEN-treated group for 16 weeks comparing to control ($P < 0.05$, Figure 3B). Otherwise, the XIAP mRNA expression was decreas-

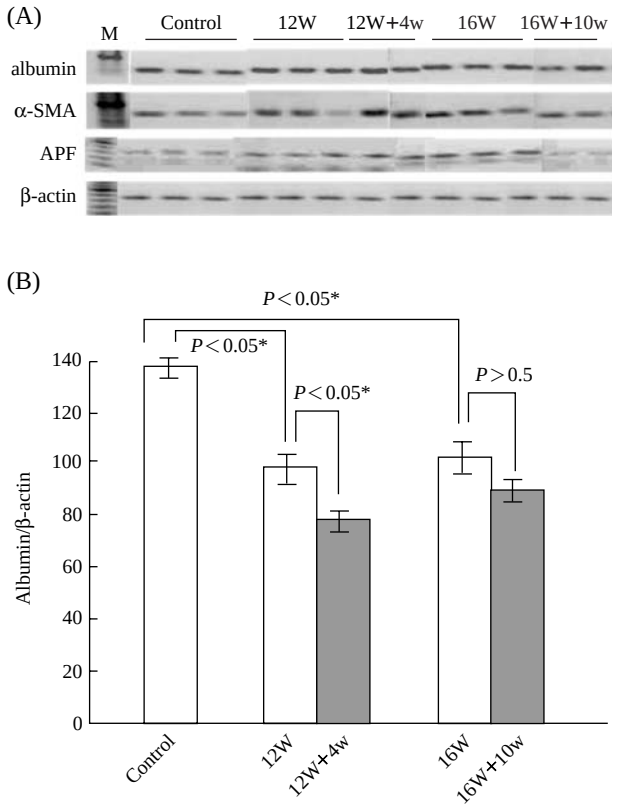

Figure 2. Expression of liver function-related genes in DEN-induced hepatocellularcarcinoma model. (A) mRNA expression of albumin, α-SMA, and APF in DEN-induced HCC model using RT-PCR. (B) Densitometric analysis of albumin mRNA expression in DEN-induced HCC relative to β-actin mRNA expression. Data are expressed at the mean ± SD. Statistical analysis was carried out by unpaired student's t-test (*$P < 0.05$).

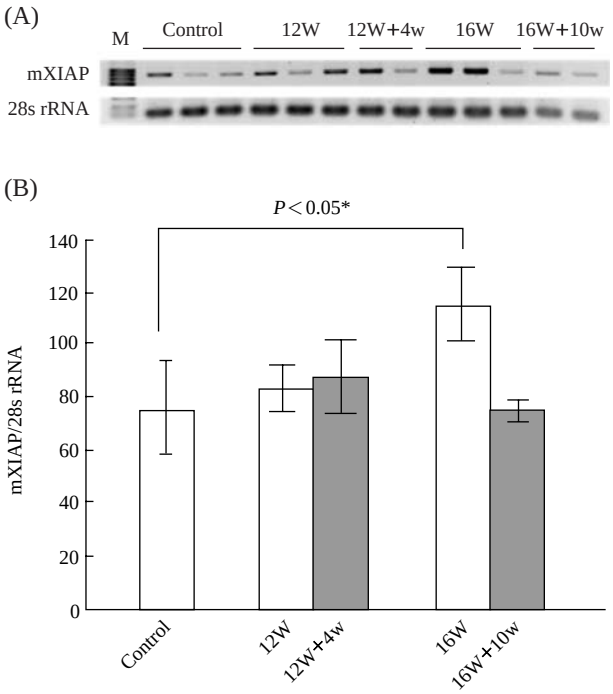

Figure 3. Expression of murine XIAP in DEN-induced hepatocellularcarcinoma model. (A) mRNA expression of murine XIAP in DEN-induced HCC model using RT-PCR. (B) Densitometric analysis of murine XIAP mRNA expression in DEN-induced HCC relative to β-actin mRNA expression. Data are expressed at the mean ± SD. Statistical analysis was carried out by unpaired student's t-test (*$P < 0.05$).

ed in DEN-treated group for 16 weeks with recovery periods rather than group without recovery periods. These results suggested that DEN-treated rat for 12 weeks with recovery periods improved the fibrosis in the liver and DEN-treated rat for 16 weeks were capable of the hepatocellular carcinoma formation. There was a necrosis showing DEN-treated liver for 16 weeks with recovery.

Histopathological Analysis of DEN-treated HCC

Periportal fibrosis was observed in the DEN-treated groups at 12 weeks and was progressed at the DEN-treated group for 16 weeks (Figure 4). Especially, the fibrosis was observed mostly around the central vessel and large fibrotic area in DEN-treated groups. Otherwise, the thickened fibrosis became thinner and shown vacuolus degeneration in DEN-treated group for 12 weeks with recovery periods. However, massive rearrangements of the fibrosis including inflammation, extensive fatty and vacuolus as well as tumor in DEN-treated group for 16 weeks with recovery periods were observed. In addition, hepatic necrosis observed after DEN-treated group for 16 week with recovery periods.

Correlation between XIAP Expression and Apoptosis Makers in DEN-induced HCC

In order to analyze the localization of XIAP in rat liver, we performed immunohistochemistry using antibody for murine specific XIAP. The expression of XIAP was observed in nuclei of hepatocyte and some cytoplasm (Figure 5A). The expression of XIAP in DEN-induced HCC was more increase DEN-treated groups with recovery periods as well as shown to increase than control (data not shown). In western analysis, the XIAP expression in DEN-treated groups gradually increased in DEN-treated groups and strong expression of XIAP was in DEN-treated for 16 weeks (Figure 5B). To confirm whether the expression of XIAP in hepatocellular carcinogenesis through DEN treatment and recovery periods correlates the regener-

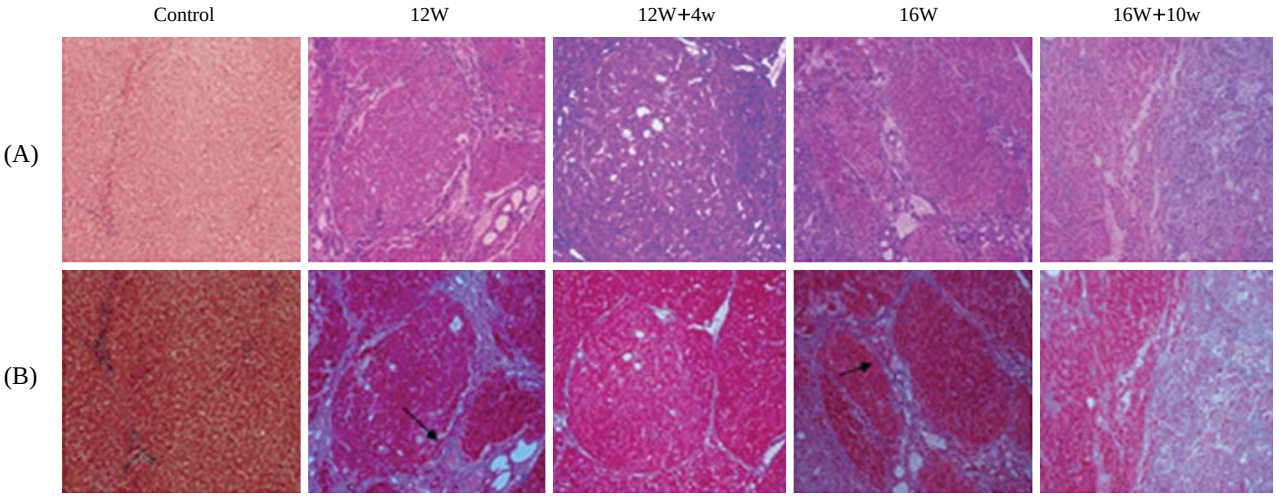

Figure 4. Histopathological analysis in DEN-induced HCC model. (A) Hematoxyline and Eosin staining (H & E) in DEN-induced HCC model. (B) Massontrichrom's staining (MT) in DEN-induced HCC model. Arrow means collagen deposition (× 100).

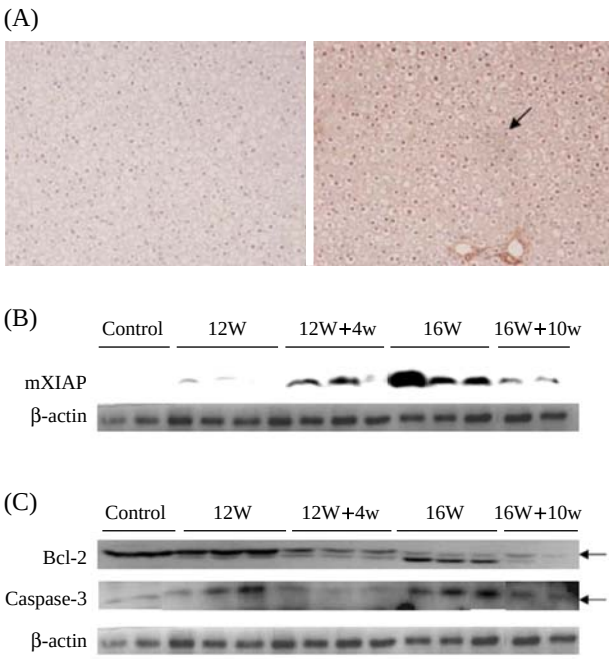

Figure 5. Expression of apoptosis-related genes and murine XIAP in DEN-induced HCC model using western blot. (A) Expression of murine XIAP in DEN-induced HCC using immunohistochemistry. Negative control (left panel) and XIAP positive (right panel) (× 200), (B) Expression of murine XIAP in DEN-induced HCC, (C) Expression of Bcl-2 and caspase-3 in DEN-induced HCC. Arrow means XIAP positive cells.

ation of hepatocyte, apoptosis-related markers were assessed in the rat liver by western blot. Figure 5C shows that the expression of Bcl-2, anti-apoptotic marker, more decreased in DEN-treated groups than control. Especially, the expression of XIAP in DEN-treated for 16 weeks with recovey periods significantly decreased. Otherwise, the expression of caspase-3 in DEN-treated groups was significantly increased than control or DEN-treated groups with recovery periods. These results suggested that the alteration of XIAP expression in DEN-induced HCC may help the hepatocyte to progress apoptosis and promote the hepatocellular carcinogenesis.

Discussion

In the present study, we demonstrated that have provided insights concerning the carcinogenesis of liver depend on DEN-treatment periods and the alteration of XIAP expression in DEN-induced HCC. The alteration of XIAP expression could be correlated to the hepatocellular carcinogenesis in DEN-induced HCC rat model.

DEN have been widely used as a hepatotoxinogen in animal models because of DEN did not induce tissue injury in other organs[21-24]. Thus, many researchers have been developed animal model to explain the DEN-induced HCC for understanding of hepatorcarcinogenesis. However, several challenges including the condition of DEN dosage, exposure timing of DEN, and susceptibility to DEN depends on animal variations still exist in generation of model for DEN-induced HCC. In addition, these models have not provided exactly markers for evaluation of the DEN-induced HCC although DEN plays a causal role in the development of HCC[25].

Hepatocellularcarcinoma (HCC) is one of common

cancer in East-North Asia[26]. Recent reports have revealed that the hepatocarcinogenesis was major responsible for the imbalance between cell proliferation and apoptosis[5,6]. Uncontrolled proliferation of cells causes expressions of various proliferation-related proteins such as PCNA, Ki-67 and Cyclin Ds through the disorganization of cell cycle[27,28]. Cell cycle-related proteins have been found to express the inflammatory and hyperplasia of cancers in human and rats[29-31]. The alterations of these proteins observed in DEN-induced HCC groups for 12 weeks and 16 weeks (data not shown).

Otherwise, apoptosis play a major in the regulation of cell numbers in many biological processes such as cell proliferation and differentiation[20,32]. Among the apoptosis-related factors, X-linked inhibitor of apoptosis (XIAP), which is the IAP family member, is the strongest inhibitor of caspases as well as a significant cancer marker[33]. However, little is known regarding the function of XIAP in carcinogenesis. In our study, XIAP expressed in the cytoplasm and discrete nuclear staining is additionally seen in DEN-induced HCC for 12 weeks and 16 weeks with or without recovery periods. Especially, XIAP expression was significantly higher in DEN-induced HCC for 16 weeks when compared that group with control. The expression of XIAP increased in DEN-induced HCC with revoery periods than without recovery periods in 12 weeks. However, the expression of XIAP was rather highly expressed in DEN-induced HCC for 16 weeks without recovery periods than those with recovery periods. These results are similar to expression of caspase-3 in DEN-induced HCC groups. The decreased expression of XIAP in DEN-induced HCC for 16 weeks with recovery periods may be cell death according to progress of necrosis in DEN-induced HCC. Sakemi and colleagues have been reported that XIAP expression is not correlated to the histological grade of HCC. However, expression of XIAP is significantly related to the poorly differentiated HCC[16]. These results suggest that the balance between cell growth and apoptosis through the expression of XIAP involved in hepatocellular carcinogenesis.

In conclusion, the alteration of XIAP expression could be present for the assessment of the DEN-induced HCC in animal model and use a marker for hepatocellular carcinogenesis.

Materials & Methods

Generation of DEN-induced Hepatocarcinogenesis Animals Model

Three-week-old male Wistar rats were purchased from Japan SLC, Japan. We conducted all animal experimental procedures using protocols approved by the National Institutes of Health Guidelines. All rat were maintained under routine condition (room temperature $22 \pm 1^{\circ}C$, humidity 45-60%) in a 12 hours light/dark cycle with free access to water and food. The rats were divided into control group and DEN-induced model group (Group I and Group II). The Group I and Group II were injected intraperitoneally with DEN dissolved in oil (50 mg/kg) twice a week for 12 weeks (Group I) and 16 weeks (Group II). After 12 weeks and 16 weeks, rats were sacrificed and their livers were collected for analysis.

RT-PCR

Rat liver tissues were homogenized and lysed in 1 mL of Trizol (Invitrogen). Total RNA (1 μg) was used to synthesize cDNA using the First-strand cDNA synthesis Kit (Invitrogen). The synthesized cDNA was amplified by use of PCR with *Taq* DNA polymerase

Table 1. Primer sequences used for RT-PCR.

Genes	Primer sequences	Tm (°C)	Size (bp)
Albumin	F: 5′-CTT CAA AGC CTG GGC AGT AG-3′ R: 5′-GCA CTG GCT TAT CAC AGC AA-3′	57	360
AFP	F: 5′-CAG TGA GGA GAA ACG GTC GG-3′ R: 5′-ATG GTC TGT AGG GCT CGG CC-3′	57	215
α-SMA	F: 5′-ACT GGG ACG ACA TGG AAA AG-3′ R: 5′-CAT CTC CAG AGT CCA GCA CA-3′	57	256
mXIAP	F: 5′-ATG ACT TTT AAC AGT TTT GAA GGA ACT-3′ R: 5′-GAA GCA CTT CAC TTT ATC GCC T-3′	54	900
28s rRNA	F: 5′-TTG AAA ATC CGG GGG AGA G-3′ R: 5′-ACA TTG TTC CAA CAT GCC AG-3′	52	100
β-actin	F: 5′- TCC TTC TGC ATC CTG TCA GCA-3′ R: 5′-CAG GAG ATG GCC ACT GCC GCA-3′	58	300

(SolGent co., Ltd, Korea). The samples were initially denatured at 94˚C for 3 minutes, followed by the thermal cycles; denaturation at 95˚C for 30 seconds, annealing at the temperature set for each pair of primers for 45 seconds, extension at 72˚C for 1 minute and the thermal cycle was repeated 35 times for all genes. Primers used for RT-PCR analysis result are shown in Table 1. The products of the PCR procedure were visualized on a 1% agarose gel containing 0.5 μg/mL ethidium bromide and photographed.

Histological and Immunohistochemical Analysis

Liver tissue was used for histological and immunohistochemical analysis. Liver tissues were fixed in 10% (v/v) buffered formaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were sliced into 5 μm thickness. The slides stained with hematoxylin and eosin (H & E). Sections for semi-quantitative analysis of fibrosis were stained using methyl blue alone in adaptation of Masson's trichrome stain (Cohen, 1976). For inmmunohistochemical analysis, deparaffinized tissue sections were treated with 3% H_2O_2 in methanol for 10 minutes to remove endogenous peroxidase followed by antigen retrieve was carried out using sodium citrate buffer (0.1 M citrate) and microwave method. Then, normal serums applied onto the slides to block any nonspecific binding. After that, the slides were incubated with primary antibody, goat IgG anti-rat XIAP (R & D systems, Minneapolis, MN, USA) diluted 1 : 200 with diluents solution at 4˚C for overnight. After further incubation with 1 : 500 diluted biotinylated goat anti-rat (DAKO, Carpinteria, CA) for 2 hours and horseradish peroxidase conjugated streptoavidin-HRP comples, the signals were detected with 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate-chromogen solution followed by conterstaining with Mayer's hematoxyline.

Western Blotting

Lysates were normalized for protein concentration, subjected to one-dimesional sodium-dodecylsulfate polyacrylamide gel electrophosresis (SDS-PAGE, 10% gradient), and proteins were electrophoretically transferred to PVDF membrane in a Bio-Rad Trans-Blot (140 mA, 90 minutes). Membranes were incubated in the blocking solution overnight at 4˚C, and then incubated with primary antibody, goat IgG anti-rat XIAP (R & D systems, Minneapolis, MN, USA) diluted 1 : 1,000 in blocking buffer for 1 hour at room temperature. Blots were washed with tris-buffered saline with tween 20 (TBS-T) following the membranes were incubated for 2 hours at room temperature with an HRP-conjugated secondary antibody diluted 1 : 1,000 in blocking buffer. After washing the blots with TBS-T, the ECL advance kit (Amersham Bioscience, Piscataway, NJ, USA) applied to the blot and developed.

Statistical Analysis

Denstoemetric analysis was used to quantify the Western blots and RT-PCR data. Data were analyzed by imageJ (Microsoft Java 1.1.4.), and group comparisons were performed using the t-test with a significance level of $P < 0.05$.

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