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Oriental Pharmacy and Experimental Medicine 2009 **9(2)**, 106-114
DOI 10.3742/OPEM.2009.9.2.106

In-Jin-Ho-Tang as a potential anti-cancer drug by induction of apoptosis in HepG2 cells

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Received for publication May 20, 2008; accepted April 22, 2009

SUMMARY

Hepatocellular carcinoma is the world's most common primary malignant tumor of the liver. In-Jin-ho-Tang (IJHT) has been used as a traditional Chinese herbal medicine since ancient times, and today it is widely used as a medication for jaundice associated with inflammation of the liver. In-Jin-Ho-Tang is a drug preparation consisting of three herbs: Artemisiae Capillaris Herba (*Artemisia capillaries* T_{HUNS}, Injinho in Korean), Gardeniae Fructus (*Gardenia jasminodes* E_{LLIS}, Chija in Korean) and Rhei radix et rhizoma (*Rheum palmatum* L., Daehwang in Korean). This study investigated whether or not methanol extract of IJHT could induce HepG2 cancer cell death. Cytotoxic activity of IJHT on HepG2 cells was measured using an XTT assay, with an IC_{50} value of 700 μg/ml at 24 h Apoptosis induction by IJHT in HepG2 cells was verified by the cleavage of poly ADP-ribose polymerase, and a decrease in procaspase-3, -8, -9. Treatment of IJHT resulted in the release of cytochrome *c* into cytosol, loss of mitochondrial membrane potential ($\Delta\Psi_m$), decrease in anti-apoptotic Bcl-2, and an increase in pro-apoptotic Bax expression. Thus, IJHT induced apoptosis in HepG2 cells via activation of caspase and mitochondria pathway. These results indicate that IJHT has potential as an anti-cancer agent.

Key words: In-Jin-ho-Tang; Apoptosis; Caspase; Mitochondria pathway; Anti-cancer drug

INTRODUCTION

Hepatocellular carcinoma (HCC) is the world's most common primary malignant tumor of the liver (Nowak *et al.,* 2004). Although substantial advances have been made in chemotherapy regimens, such as doxorubicin for HCC (Zhu *et al.,* 2006), the efficacy of drugs is often hampered by adverse side effects. Therefore, a new approach for development of an effective therapy against this disease is needed.

Apoptosis, implicated in a variety of physiological processes. It is characterized by a number of well-defined features, such as cellular morphological change, membrane blebbing, and activation of a family of cysteine proteases called caspase and so on. (Thornberry, 1998a). Activated caspase-8 can activate downstream caspases either by direct cleavage, or by indirectly cleaving Bid and inducing cytochrome *c* release from the mitochondria. In the mitochondrial-initiated pathway, caspase activation is triggered by the formation of a multimeric Apaf-

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1/cytochrome *c* complex that is fully functional in activating procaspase-9 (Budihardjo *et al.,* 1999). Caspase-3 has been shown to cleave many substrates, including poly (ADP-ribose) polymerase (PARP), that respond to DNA strand breaks and consequently lead to apoptosis (Thornberry *et al,* 1998b).

A number of studies have shown that administration of herbal medicine leads to regulation of many different types of immune responses. For example, herbal medicines can decrease severity of hepatic fibrosis (Wasser *et al.,* 1998; Lee *et al.,* 2003; Lee *et al.,* 2007), inhibit the invasiveness potential of HCC (Ha *et al.,* 2004a), inhibit proliferation of smooth muscle cells (Ha *et al.,* 2004b), or increase immunosuppressive effects (Shao *et al.,* 2007). Therefore, the authors investigated the effects of an oriental medicinal prescription, In-Jin-ho-Tang (IJHT; Yin-chen-hao-tang in Chinese and Inchin-ko-to in Japanese/ Japanese herbal medicine TJ-135) (Iinuma *et al.,* 2003; Lee *et al.,* 2007). The medication has long been used in China, Japan and Korea as an anti-inflammatory, antipyretic, choleretic and diuretic agent for liver disorders and jaundice, and several studies provide clinical evidence of its effectiveness in the treatment of various liver diseases. In addition, it has purgative, anti-viral, and anti-bacterial effects (Yamashiki *et al.,* 2000; Iinuma *et al.,* 2003).

IJHT has been reported to show a potent inhibitory effect on hepatocyte apoptosis induced by transforming growth factor beta 1 (TGF-β1), and it inhibits the production of inflammatory cytokines, interleukin-12 and interferon-γ in concanavalin A-induced hepatitis (Yamamoto *et al.,* 1996; Yamashiki *et al.,* 2000). Also, when mice were pretreated with oral IJHT for 1 week before con A injection, the activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were significantly decreased after con A administration. IJHT also promotes bile secretion (Sakagami *et al.,* 1985). Furthermore, IJHT has been reported to directly suppress liver fibrosis *in vivo*, and ameliorate hepatic fibrosis in bile duct ligation rats (Inao *et al.,* 2004; Lee *et al.,* 2007). Clinical reports in Japan, IJHT is sometimes used to treat patients with primary biliary cirrhosis (Onji *et al.,* 1990) or hepatitis C (Itoh *et al.,* 1997). However, little is known about the anti-cancer activity of IJHT or its signal pathway against hepatocellular carcinoma. In this study, the authors hypothesized that methanol extract of IJHT has potent cytotoxicity on human hepatocellular carcinoma HepG2 via activation of caspase and mitochondria pathway.

MATERIALS AND METHODS

Reagents

An XTT kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Aprotinin, leupeptin, RNase A, DMSO, dithiothreitol (DTT), sodium orthovanadate (SOV), SB203580, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K was purchased from Invitrogen (Carlsbad, CA). Anti-poly ADP-ribose polymerase (PARP), procaspase-9, phospho-ERK 1/2, phospho-p38, and β-actin monoclonal antibody (mAb) were purchased from Cell Signaling Technology (Beverly, MA). Anti-procaspase-3, procaspase-8, ERK 1/2, p38 and Bax mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG horseradish peroxidase (HRP), anti-mouse IgG HRP and Anti-goat IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome *c* and Bcl-2 mAb were purchased from BD PharMingen (San Jose, CA).

Preparation of methanol (MeOH) extract from IJHT

A prescription formulation of In-Jin-ho-Tang (IJHT) was obtained from Dongguk University Oriental Medical Hospital, Kyungju, Korea. In addition, for laboratory study, authentic plant materials were purchased from a local market and identified at the Oriental Medical Department, Dongguk University, Kyungju, Korea.

IJHT extract powder consists of crude ingredients extracted from the following three medicinal herbs: Artemisiae Capillaris Herba (*Artemisia capillaries* T_{HUNS} Injinho in Korean), Gardeniae Fructus (*Gardenia jasminodes* E_{LLIS}, Chija in Korean) and Rhei radix et rhizoma (*Rheum palmatum* L., Daehwang in Korean) with a weight ratio of 10:5:3. IJHT was extracted with 80% methanol for 48 h at room temperature. The extract was filtered and concentrated using a rotary evaporator, then freeze-dried using a freeze dryer to obtain 48.06 g of methanol extract of IJHT (yield, 17.8%). The methanol extract was dissolved in Dulbecco's modified Eagle's medium (DMEM, HyClone).

Cell culture

The human hepatocellular carcinoma cell line HepG2 and human liver cell line Chang were obtained from the Korean Cell Line Bank (Seoul, South Korea) and the American Type Culture Collection (Rockville, MD, USA), respectively. The cells were maintained in a monolayer culture at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin).

XTT assay

The cytotoxic activity of natural products was measured as previously described (Ha *et al.,* 2004a). The extract of IJHT was examined for cytotoxic activity on HepG2 cells using a commercially available proliferation kit, XTT II. Briefly, the cells were plated in 96-well culture plates at a density of 3×10^4 cells/well in DMEM culture medium and allowed to attach for 24 h. The extracts were added to various final concentrations (0~700 μg/ml, and control: 0 μg/ml) in triplicate. After 24 h of culture, 50 μl of XTT reaction solution (sodium 3-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate, mixed in 50:1 proportion) was added to the wells. The optical density was read at a wavelength of 450 nm in an ELISA plate reader after a 4 h incubation of the plates at 37 °C with XTT.

Analysis of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by the retention of $DiOC_6(3)$. The cationic lipophilic fluorochrome $DiOC_6(3)$ is a cell permeable marker that specifically accumulates into mitochondrion depending on $\Delta\Psi_m$ (Lin *et al.,* 2003). HepG2 cells (5×10^5) were treated with various concentrations of IJHT for 24h. Cells were washed once in 0.2% BSA-PBS and then incubated with 100 nM $DiOC_6(3)$ for 30 min at 37 °C. As many as 1×10^4 cells were analyzed at FL1 (530 nm) with a Becton Dickinson FACScan flow cytometer and CELL-Quest software (BD Biosciences, San Jose, CA).

Determination of apoptosis by flow cytometry

To detect externalized phosphatidylserine (PS) as an early indication of apoptosis, HepG2 cells (1×10^6/ml) were incubated with various concentrations of IJHT for 24 h at 37 °C. Cells were then harvested, stained with Annexin V-FITC according to the manufacturer's protocol (Clontech Laboratories, Palo Alto, CA), and analyzed with a Becton Dickinson FACScan flow cytometer and CELL-Quest software (BD Biosciences, San Jose, CA).

To examine cell cycle distribution, HepG2 cells (1×10^6/ml) were washed with ice-cold PBS and fixed in 70% ethanol at -20 °C overnight. After fixation, cells were washed in 0.2% BSA-PBS. Cells were stained in 0.5 ml PI-RNase solution (BD Pharmingen, San Jose, CA) for 30 min in the dark at room temperature. Fluorescence emitted from the propidium-DNA complex was detected with a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Preparation of cytosolic extractions for cytochrome *c* analysis

The cells were washed with ice-cold PBS and resuspended in cold lysis buffer (250 mM sucrose,

20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 30 min, cells were homogenized with 15 strokes in a douncer, and the homogenates were centrifuged at 1,200 g for 15 min at 4 ºC. The supernatants were transferred to another tube and centrifuged at 100,000 g for 60 min at 4 ºC and loaded on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cytochrome *c* release was analyzed by immunoblotting using an anti-cytochrome *c* mAb.

Preparation of total cell extracts and Western blotting

HepG2 cells were washed three times with ice-cold PBS and harvested by centrifugation. The cells were sonicated in 50 mM Tris-HCl buffer (pH7.5, 5 mM $MgCl_2$, 5 mM NaCl, 5% (v/v) glycerol, 20 mM NaF, 2 mM Na_3VO_4 and various protease inhibitors). After centrifugation at 12,000 g for 15 min at 4 ºC, supernatants were collected and referred to cell extract. The protein concentrations were measured using the Bradford protein dye reagent (Bio-Rad). Total cell lysates from HepG2 cells were separated by 10%~12.5% SDS-PAGE. Proteins were transferred electrophoretically onto nitrocellulose membranes (0.2 μm, Schleicher and Schuell). The membranes were blocked with 5% non-fat dried milk in Tris buffered saline (TBS) and were subsequently probed with primary antibody in TBS containing 3% non-fat dried milk. Antibody-antigen complexes were detected using goat anti-mouse IgG or goat anti-rabbit IgG-HRP conjugated antibodies and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, UK, USA).

Statistics

The data shown are the mean ± S.E.M. Differences between groups were evaluated by Student's *t*-test. The results presented are representative of at least three independent experiments.

RESULTS

Cytotoxic effect of IJHT in hepatic cell lines HepG2 and Chang

The cytotoxicity of IJHT on HepG2 cells was assessed by XTT assay. Cells were incubated for 24 h in 96-well microplates either with or without various concentrations of IJHT (0, 50, 100, 300, 500 and 700 μg/ml). Under the experimental conditions, IJHT treatment resulted in marked inhibition of the survival of HepG2 cells in a dose-dependent manner (Fig. 1). IJHT has weak cytotoxicity for HepG2 cells. From Figure 1, the IC_{50} value (concentrations required for 50% growth inhibition) of IJHT was 700 μg/ml at 24 h. The most prominent inhibitory activity of IJHT on HepG2 cell survival was attained with a 24 h culture.

To determine whether or not the compound exerted its action specifically on the tumor cells, a human normal liver cell line, Chang, was included as a parallel experiment. Fig. 1 shows that more than 90% of cells were still viable when treated with the effective dosage, 700 μg/ml, against HepG2 cancer cells.

IJHT induces apoptosis in HepG2 cells

Cells undergoing apoptosis show characteristic changes such as nuclear condensation, DNA

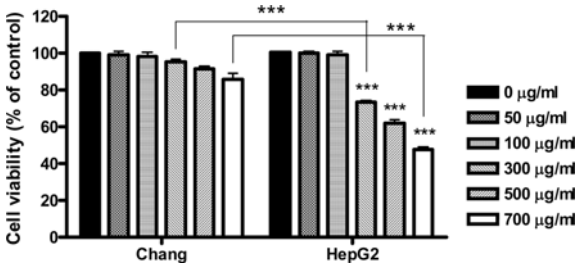

Fig. 1. Cytotoxicity of IJHT in hepatic cells HepG2 and Chang. HepG2 and Chang cells were seeded in 96-well plates and incubated with IJHT. HepG2 and Chang cells were exposed to the indicated concentration of IJHT for 24 h (IC_{50} = 700 μg/ml). Cell viability was determined by XTT assay. Data shown are representative of at least three independent experiments. Data are means ± S.E.M. ***P < 0.001 compared with the control group (IJHT, 0 μg/ml).

fragmentation, and translocation of PS to the outer leaflets of the plasma membrane (Wyllie *et al.*, 1980; Hur *et al.*, 2004). Apoptosis was measured by annexin V staining of PS following IJHT treatment. HepG2 cells were incubated with various concentrations of IJHT (0 - 700 μg/ml) for 24 h. IJHT markedly induced apoptosis in HepG2 cells at a concentration of 700 μg/ml (Fig. 1A; 0 μg/ml, 5.5 ± 0.8%; 50 μg/ml, 13.3 ± 0.8%; 100 μg/ml; 19 ± 3.9%; 300 μg/ml, 19.3 ± 2.7%; 500 μg/ml, 20.7 ± 2.8%; 700 μg/ml, 38.5 ± 4%; respectively).

To confirm IJHT-induced apoptosis, we also performed a flow cytometric analysis after PI staining. HepG2 cells were incubated with various concentrations of IJHT (0 - 700 μg/ml) for 24 h. Then 3.8 ± 1.5% and 34.3 ± 3.6% DNA fragmentation, represented by the percentage of cells with hypodiploid DNA, was observed in the control cells (IJHT, 0 μg/ml) and the IJHT-treated cells (700 μg/ml), respectively (Fig. 1C). Figure 1 shows that 35 - 40% of HepG2 cells displayed apoptosis, after 24 h of IJHT treatment.

IJHT induces poly (ADP-ribose) polymerase cleavage and caspase-3, -8, -9 activation

To confirm further the apoptosis induced by IJHT, the authors investigated the cleavage of PARP, which responds to DNA strand breaks and is used as another hallmark of apoptosis. As shown in Figure 3A, the cleavage of PARP was detected after 24 h of exposure to 300 μg/ml of IJHT. The most prominent PARP activity of IJHT in HepG2 was attained at 700 μg/ml concentration (Fig. 3A). It has been reported that caspase-3, -8, and -9 play a pivotal role in the terminal phase of apoptosis (Lin S *et al.*, 2003), so the activation of these caspases induced by IJHT was investigated. As shown in Figure 3B-D, IJHT treatment induced the activation of caspase-3, -8, and -9 in a dose-dependent manner at 24 h. The appearance of cleavage forms of the three caspases was observed at 300 μg/ml after the addition of IJHT. These data suggest that a caspase-dependent pathway is involved in IJHT-induced apoptosis.

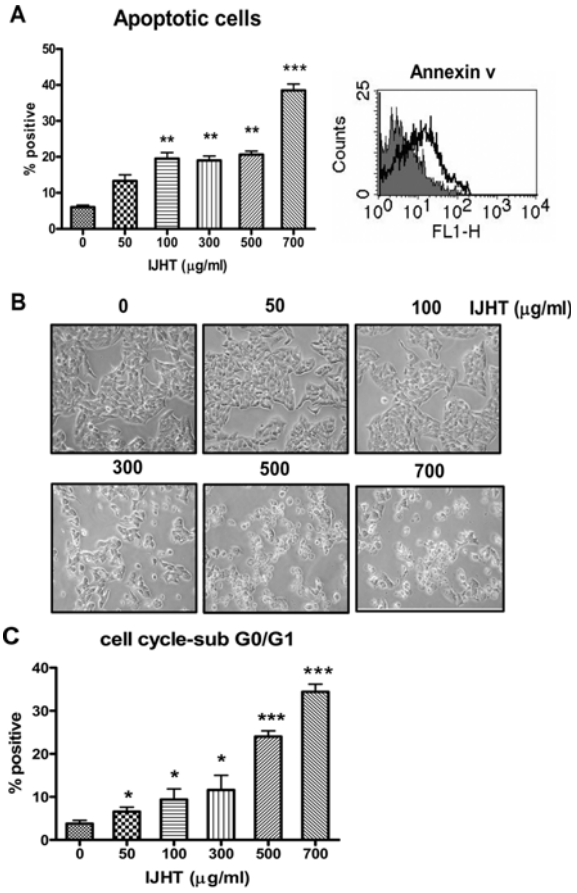

Fig. 2. IJHT induces apoptosis in HepG2. HepG2 cells incubated to the indicated concentrations of IJHT for 24 h. (A) Dose-dependently induced apoptotic cells. Apoptosis as measured by staining with FITC-labeled annexin V, followed by flow cytometric analysis and the percentage of apoptotic cells. (B) IJHT-induced morphological changes of HepG2. The cells were photographed under a microscope (magnification × 200). (C) Dose-dependently induced sub G0/G1 phase cells. DNA fragmentation as measured by flow cytometric analysis and the percentage of sub G0/G1 phase cells. Data shown are representative of at least three independent experiments and are means ± S.E.M. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ compared with the control group.

IJHT induces cytochrome *c* release from mitochondria loss of mitochondria membrane potential, decreased Bcl-2 and increased Bax protein

Mitochondria play a very important role in

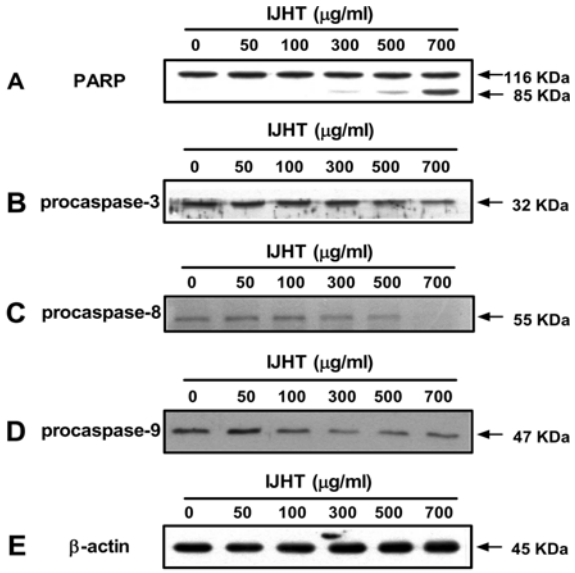

Fig. 3. IJHT induces PARP cleavage and caspase-3, -8, -9 activation. HepG2 cells were treated with IJHT at the indicated dosage for 24 h. Whole-cell lysate was obtained and used for Western blotting analysis with various specific antibodies, respectively, as described in Materials and Methods. (A) Cleavage of PARP. (B) Caspase-3 activation. (C) Caspase-8 activation. (D) Caspase-9 activation. (E) β-actin.

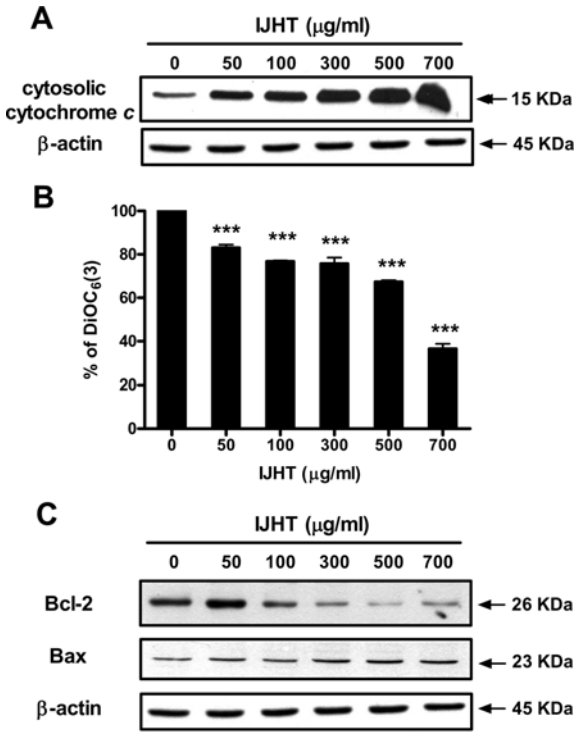

Fig. 4. IJHT induces cytochrome *c* release from mitochondria, the loss of mitochondria membrane potential ($\Delta\Psi_m$), a decreased Bcl-2 and an increased Bax protein. Cytochrome c release. HepG2 (1×10^6 cells/ml) cells were treated with various concentrations of IJHT (0, 50, 100, 300, 500 and 700 μg/ml) for 24 h. Cytosolic and mitochondrial fractions were isolated, and cytochrome c was determined by using cytochrome c antibody as described in Materials and Methods. (B) Loss of $\Delta\Psi_m$. HepG2 (1×10^6 cells/ml) cells were incubated with various concentrations of IJHT (0, 50, 100, 300, 500 and 700 μg/ml) for 24 h., Cells were then incubated with 100 nM $DiOC_6(3)$ for 30 min, followed by flow cytometric analysis as described in Materials and Methods. (C) Protein levels of Bcl-2 and Bax. HepG2 (1×10^6 cells/ml) cells were incubated with various concentrations of IJHT (0, 50, 100, 300, 500 and 700 μg/ml) for 24 h. Whole cell lysate was obtained and used for Western blotting analysis with Bcl-2 and Bax antibodies. Data shown are representative of at least three independent experiments. Data are means ± S.E.M. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ compared with the control group.

apoptosis (Petit *et al.,* 1997). It has been shown that translocation of cytochrome *c* from the mitochondrion is an essential event in apoptotic signaling, which then causes apoptosis by caspase activation (Singh *et al.,* 2005). As shown in Fig. 4A, a dose-dependent accumulation of cytochrome *c* in the cytosol was detected in IJHT-treated cells, and the efflux in mitochondrion was observed simultaneously. This was due to cytochrome *c* release linked to the loss of mitochondria membrane potential ($\Delta\Psi_m$) (Garland *et al.,* 1998). We next examined the effect of IJHT on the $\Delta\Psi_m$ by using potential-sensitive dye $DiOC_6(3)$. A remarkable loss of $\Delta\Psi_m$ was observed at 24 h, at 700 μg/ml of IJHT (Fig. 4B). The results suggest that IJHT causes an efflux of cytochrome *c* from mitochondrion by a loss of ΔY_m. Furthermore, the protein expression pattern of the Bcl-2 family was also measured. As shown in Fig. 4C, the pro-apoptotic protein Bax increased as the concentration of IJHT increased. By comparison, the anti-apoptotic protein Bcl-2 decreased as the concentration of IJHT increased. These data suggest

that IJHT-induced apoptosis might be regulated by mitochondrial disorganization.

DISCUSSION

The herbal medicine In-Jin-Ho-Tang induces apoptosis in human hepatoma HepG2 cells. IJHT induces apoptotic cells and a sub-G0/G1 population (Fig. 2), PARP cleavage and caspase activation (Fig. 3), cytochrome c release from mitochondria and loss of $\Delta\Psi_m$, and, a decreased Bcl-2 and an increased Bax protein level (Fig. 4). It is hypothesized that the IJHT-treated HepG2 cells were killed by apoptosis, promoting PARP cleavage, caspase activation, releasing cytochrome c from mitochondria and loss of $\Delta\Psi_m$ (mitochondrial disorganization), decreasing survival protein Bcl-2, and increasing apoptotic protein Bax.

IJHT-induced apoptosis occurred by down-regulation of the anti-apoptotic protein Bcl-2 and by up-regulation of the pro-apoptotic protein Bax (Fig. 4). Also, it induced inactivation of ERK1/2 (the level of phosphorylation was decreased), as well as phosphorylated Akt and Raf (data not shown). IJHT-induced cell death occurred by inhibiting cell growth signals such as the Ras/Raf/MEK/ERK pathway (decreased p-ERK1/2, p-Akt and p-Raf activity) and by activating the apoptotic pathway that accelerates apoptosis of IJHT-treated HepG2 cells. Thus, IJHT-induced cell death of HepG2 was controlled by Ras/Raf/MEK/ERK signaling pathway.

IJHT, a folk medication, has long been used in China, Japan and Korea as an anti-inflammatory, antipyretic, choleretic and diuretic agent for liver disorders and jaundice. Several studies provide clinical evidence of its effectiveness in the treatment of various liver diseases. In addition, it has purgative, anti-viral, and anti-bacterial effects (Yamashiki *et al.,* 2000; Iinuma *et al.,* 2003).

Recently, IJHT has been reported to show a potent inhibitory effect on the hepatocyte apoptosis, and it inhibits the production of interleukin-12 and interferon-γ in concanavalin A-induced hepatitis (Yamamoto *et al.,* 1996; Yamashiki *et al.,* 2000). Also, when mice were pretreated with oral IJHT for 1 week before con A injection, the activities of serum AST, ALT and LDH were significantly decreased after con A administration (82%, 96% and 66% respectively). IJHT also promotes bile secretion (Sakagami *et al.,* 1985). Furthermore, IJHT has been reported to directly suppress liver fibrosis *in vivo*, and ameliorate hepatic fibrosis in bile duct ligation rats (Inao *et al.,* 2004; Lee *et al.,* 2007). Clinical reports in Japan, IJHT is sometimes used to treat patients with primary biliary cirrhosis (Onji *et al.,* 1990) or hepatitis C (Itoh *et al.,* 1997). However, little is known about the anti-cancer activity of IJHT or its signal pathway against hepatocellular carcinoma, although Rhubarb and its anthraquinone components, emodin, aloe-emodin and rhein have been reported to be anticancer agents (Huang *et al.,* 2006). Furthermore, genifin was isolated from Gardeniae Fructus, as a single compound enhancing bile formation (Shoda *et al.,* 2004). Therefore, further detailed investigation of other components from IJHT such as Gardeniae Fructus, Artemisiae Capillaris Herba, and genifin on anticancer activity led to findings of cancer cell cytotoxicity.

However, little is known about the anti-cancer activity of IJHT or its signal pathway against hepatocellular carcinoma, although Rhubarb and its anthraquinone components, emodin, aloe-emodin and rhein have been reported to be anticancer agents (Huang *et al.,* 2006). Furthermore, genifin was isolated from Gardeniae Fructus, as a single compound enhancing bile formation (Shoda *et al.,* 2004). Therefore, further detailed investigation of other components from IJHT such as Gardeniae Fructus, Artemisiae Capillaris Herba, and genifin on anticancer activity led to findings of cancer cell cytotoxicity.

In conclusion, the present findings suggest that methanol extract of IJHT has potent cytotoxicity on human hepatocellular carcinoma HepG2 via activation of caspase and mitochondria pathway.

IJHT is a potential anti-cancer drug for liver cancer patients.

ACKNOWLEDGEMENTS

This work was supported by the Dongguk University Research Fund and the MRC program of MOST/ KOSEF (grant #: R13-2005-013-01000-0).

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